SHARP-1 REPRESSES MYOGENIC DIFFERENTIATION THROUGH RECRUITMENT OF METHYLTRANSFERASE G9A

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Ling Mei Tze Belinda

30 June 2012

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SUMMARY

Skeletal muscles are made up of bundles of muscle fibers formed from the fusion and differentiation of myoblasts (muscle cells) into myotubes (multi-nucleated myoblasts). The process of generating muscle, known as myogenesis, is tightly regulated by basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin, and MRF4**.** MRFs control the transcriptional cascade of myogenic genes necessary for skeletal cell differentiation. The mechanisms involving MRFs in the development of the muscle fibers are well defined. However, several factors can negatively regulate myogenesis and the mechanisms by which they function in myogenesis are not clearly understood. Moreover, the mechanisms by which inhibitory factors contribute to myogenic defects and their roles in muscle disorders remain to be identified.

Sharp-1, a basic helix-loop-helix (bHLH) transcription factor, functions as a potent repressor of skeletal muscle differentiation and is dysregulated in muscle pathologies. However, the mechanisms by which Sharp-1 inhibits myogenic differentiation are unclear. In this work, I have identified a lysine methyltransferase G9a as a novel co-factor that directly associates with Sharp-1, and is critical for Sharp-1-mediated repression of muscle differentiation. Similar to Sharp-1, G9a itself has an inhibitory role in skeletal myogenesis. In addition to mediating histone H3 lysine 9 di-methylation repression marks on muscle promoters, G9a methylates MyoD, a key transcription factor required for muscle development, at lysine (K104). Overexpression of Sharp-1 in muscle precursor cells results in G9a-dependent histone modifications and MyoD methylation. siRNA-mediated knockdown of G9a or pharmacological blockage of its activity partially rescues the differentiation defects

imposed by Sharp-1. These findings provide new insights into Sharp-1-dependent regulation of myogenesis and identify epigenetic mechanisms which could be targeted in myopathies characterised by elevated Sharp-1 or G9a levels.

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List of Symbols and Abbreviations

6E-TATA-Luc	- A firefly luciferase reporter plasmid harboring Sharp-1 binding sites
AIDS	- Acquired immune deficiency syndrome
bFGF	- Basic fibroblast growth factor
bHLH	- Basic helix-loop-helix
BIX-01294	- A G9a methyltransferase inhibitor
Blimp-1	- B-lymphocyte-induced maturation protein 1
BMD	- Becker's Muscular Dystrophy
BMP	- Bone morphogenetic proteins
BrdU	- Bromodeoxyuridine
C2C12	- Mouse myoblast cell line
C3H 10T1/2	- Mouse embryonic mesenchymal cell line
CaMK	- Calcium/calmodulin-dependent protein kinase
CBP	- CREB binding factor
cDNA	- Complementary DNA
CDK2, CDK4	- Cyclin-dependent kinases
CDP/cut	- CCAAT displacement protein
CDYL1	- Chromodomain Y-like protein
C/EBPα	- CCAAT/enhancer binding protein α and α
C/EBPβ	- CCAAT/enhancer binding protein α and β
CENP-A, -B,-C	- Centromere protein A, Centromere protein B, Centromere protein C
ChIP	- Chromatin immunoprecipitation
COS7	- African Green Monkey SV40-transformed kidney fibroblast cell line
DAPI	- 4',6-diamidino-2-phenylindole

Mdx	- an animal model of Duchenne muscular dystrophy
DEC1	- Differentiated embryo chondrocyte protein 1
DEC2	- Differentiated embryo chondrocyte protein 2
DM	- Differentiation Medium
DMD	- Duchenne Muscular Dystrophy
DMEM	- Dulbecco's Modified Eagle Medium
DMSO	- Dimethyl sulfoxide
DTT	- Dithiothreitol
E12/E47/HEB	- E-proteins
E2F	- Elongation Factor 2
EDTA	- Ethylenediaminetetraacetic acid
EGFP	- Enhanced Green Fluorescent Protein
ERK	- Extracellular signal- regulated kinase
Ezh2	- Enhancer of Zeste homolog 2
FBS	- Fetal bovine serum
FGF	- Fibroblastic growth factor
FITC	- Fluorescein isothiocyanate, a green fluorescent dye
G9a	- EHMT2, Euchromatic histone-lysine N-methyltransferase 2
GAPDH	- Glyceraldehyde 3-phosphate dehydrogenase
GLP	- G9a-like protein, EHMT1, Euchromatic histone-lysine N-methyltransferase 1
GM	- Growth Medium
GST	- Glutathione S-transferase
H3K4	- Histone 3 lysine 4
H3K9	- Histone 3 lysine 9
H3K17	- Histone 3 lysine 17

H3K9me2 - Di-methyl lysine 9 of histone 3

H3K9K14ac - Acetylated lysine 9 and lysine 14 of histone 3

HAT - Histone acetyltransferase

HDAC 1,4,5 - Histone deacetylase 1, 4, 5

HEK293T - Human Embryonic Kidney 293 cell line

Hes1 - Hairy and Enhancer of Split 1

Hesr - Hairy and enhancer of split related Hesr family

Hey1 - Hairy/ enhancer-of-split-related with YRPW motif 1

HGF - Hepatocyte growth factor

HKMT - Histone Lysine Methyltransferase

HLH - Helix loop helix

Id - Inhibitor of DNA binding/differentiation

IF - Immunofluorescence

IFNB1 - Interferon, beta 1

IGF-I - Insulin-like growth factor-I

IL-6, IL-15 - Interleukin 6, Interleukin 15

IMR-90 - Human diploid fibroblast cell line

IP - Immunoprecipitation

IPTG - Isopropyl-beta-D-thiogalactopyranoside

LB - Luria-Bertani broth

MCK - Muscle creatine kinase

MEF2 - Myocyte enhancer factor-2

MEK - Mitogen-activated protein kinase

MHC - Myosin Heavy Chain

Mrf4 - Myogenic regulatory factor 4

Myf5	- Myogenic factor 5
MyoR	- Myogenic repressor
NaCl	- Sodium chloride
NF-κB	- Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH3T3	- Mouse embryonic fibroblast cell line
NRSF	- Neuron-Restrictive Silencer Factor
OD	- Optical density
p21	- Cyclin-dependent kinase inhibitor 1
P300	- Histone acetyltransferase
Pax 3, 7	- Transcription factors paired box 3, 7
PBS	- Phosphate Buffered Saline
P/CAF	- p300/CBP-associated factor
PCNA	- Proliferating cell nuclear antigen
PCR	- Polymerase chain reaction
PMSF	- Phenylmethanesulfonylfluoride
pMyogLuc	- A myogenin promoter-driven firefly luciferase reporter plasmid
PPARγ	- Peroxisome proliferator-activated receptor γ
PRDI-BF1	- Positive Regulatory Domain I-Binding Factor 1
Q-PCR	- Quantitative real-time polymerase chain reaction
Rb	- Retinoblastoma
REST	- RE1-Silencing Transcription factor
RIPA	- Radioimmunoprecipitation assay
RT-PCR	- Reverse transcriptase-polymerase chain reaction
SDS-PAGE	- Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sharp-1	- Enhancer-of-split and hairy-related protein 1

SHH	- Sonic Hedgehog
siG9a	- A siRNA directed at methyltransferase G9a
SMA	- Spinal muscular atrophy
Sp1	- Specificity Protein 1, transcription factor
Stra13	- Stimulated by retinoic acid 13
Suv39h1	- Suppressor of variegation 3-9 homolog 1, a histone methyltransferase
Texas-red	- Sulforhodamine 101 acid chloride, a red fluorescent dye
TGF-β	- Transforming growth factor
TRF1,2,3	- Telomeric repeat binding factor
Tris-HCL	- TRIS hydrochloride
TSA	- Trichostatin A
UHRF1	- Ubiquitin-like, containing PHD and RING finger domains, 1
VEGF	- Vascular endothelial growth factor
WIZ	- Widely interspaced zinc finger motifs protein
WRPW	- Tryptophan, Arginine, Proline, Tryptophan
YRPW	- Tyrosine, Arginine, Proline, Tryptophan
YY1	- Transcriptional repressor protein

Chapter 1

Introduction

1. Introduction

The development of skeletal muscle tissue, also known as myogenesis, occurs embryonically. It involves the specification of cells into skeletal muscle lineage, migration of myoblasts (muscle precursor cells), followed by differentiation and fusion of these myoblasts into myotubes (multinucleated muscle cells). Myogenesis also occurs in the adult skeletal muscle in response to damage or injury. Myogenesis is governed by myogenic regulatory factors (MRFs) which tightly control the transcriptional regulatory networks in muscle cells. A deficiency of MRFs, or any abnormality in MRF function will affect proper skeletal muscle differentiation, leading to defects in embryonic muscle development and postnatal muscle regeneration. MRFs do not work alone but act together with multiple positively and negatively-acting regulatory proteins to control myogenesis. However, the mechanisms by which MRFs activity is modulated by its associated inhibitors are still poorly understood. Therefore, identification and characterization of proteins that inhibit MRF activity are required for in-depth understanding of the molecular mechanisms in muscle development and muscle disorders. A better understanding of how the myogenic regulatory network functions will facilitate the development of effective therapies for muscle-related disorders.

1.1. Embryonic muscle development

In vertebrates, the development of skeletal muscle tissues begins from the specification, migration, proliferation and differentiation of progenitor cells in somites present at the embryo stage. In the event of muscle differentiation in embryo, environmental signals and cues stimulate some of these progenitor cells to commit into different cell lineages such as skeletal muscle lineage and neural lineage.

The embryo which consists of totipotent cells form three germ layers of progenitor cells:

1. Mesoderm are committed cells which will become the skeletal muscle, skeleton, dermis of skin, connective tissue, urogenital system, heart, blood (lymph cells), kidney, and spleen.
2. Ectoderm are committed cells which will become the central nervous system, lens of the eye, cranial and sensory, ganglia and nerves, pigment cells, head connective tissues, epidermis, hair, and mammary glands.
3. Endoderm are committed cells which will become the stomach, colon, liver, pancreas, urinary bladder, lining of the urethra, epithelial parts of trachea, lungs, the pharynx, thyroid, parathyroid, and intestines.

The mesoderm layers eventually give rise to paraxial mesoderm, neural tube and notochord. Thereafter, paraxial mesoderm separates into blocks known as somites and these somites will subsequently proliferate and differentiate into four different cells including the sclerotome (that forms the vertebrae and rib cartilage), myotome (that forms the musculature of the back, ribs and limbs), dermatome (that forms the skin on the back) and syndetome (that forms the tendons and some blood vessels) (Figure 1.1).

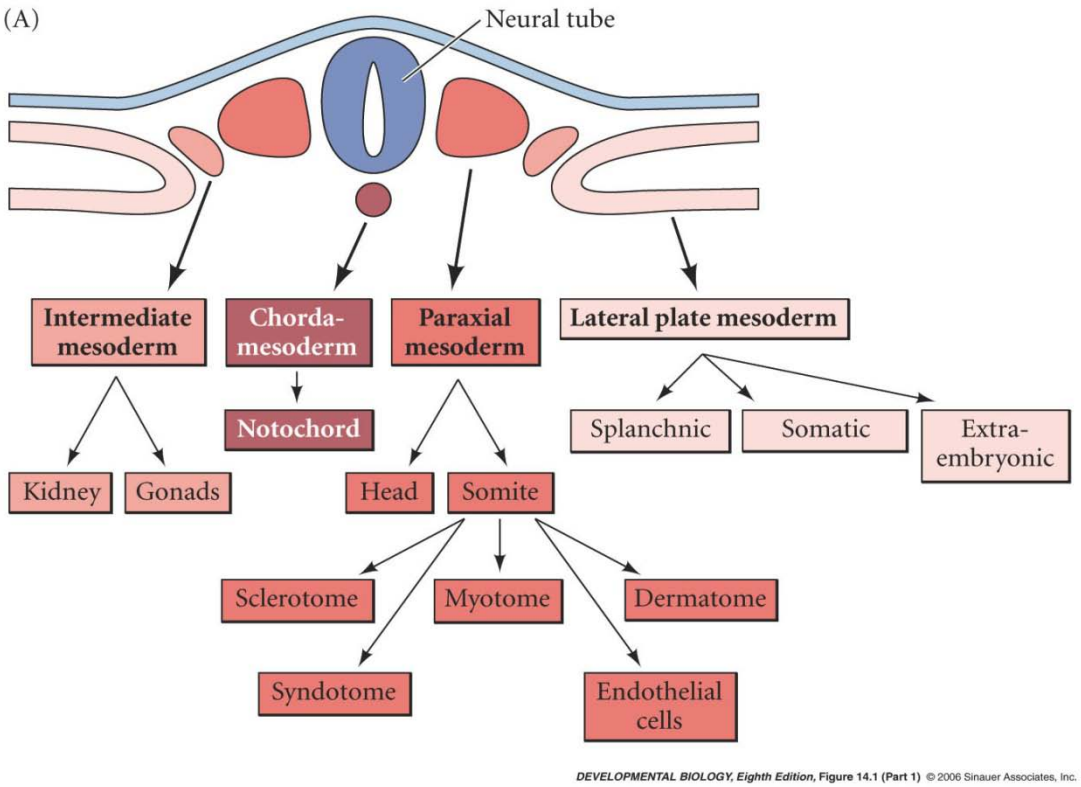

Figure 1.1. Development of muscle tissue (Taken from Scott F. Gilbert, 2010).

Subsequent to myotome and dermatome formation, committed muscle progenitor cells, known as myoblasts, will eventually migrate and generate limb musculature. After migration into the limb bud, myoblasts begin to proliferate in the presence of growth factors (Christ *et al*., 1977, Christ and Brand-Saberi, 2002). Following the depletion of growth factors, myoblasts stop dividing and secrete fibronectin onto their extracellular matrix, resulting in terminal differentiation with fusion of myoblasts into myotubes (Olson, 1992). Myotubes mature into multinucleated myofibers and muscle tissues. The proliferation and differentiation of myogenic cells and fusion of cells to existing myofibers continue during postnatal growth of muscle.

1.1.1. The myogenic regulatory network in embryonic muscle development - commitment of progenitor cells into myoblasts

The commitment of the embryonic stem cells to become muscle lineage-committed myoblasts, is controlled by microenvironmental factors such as signalling proteins, growth factors and somite segmentation. Sonic Hedgehog (SHH), a protein secreted from notochord and ventral floor plate of the neural tube, acts as one of the positive microenvironmental factors. It induces the expression of myogenic transcription factors Paired box (Pax**)** genes in the segmental plate and dermomyotome of cells (Fan and Tessier-Lavigne, 1994, Johnson *et al*., 1994, Fan *et al*., 1995). Pax genes activate their downstream targets MyoD and Myf5 which trigger the myogenic pathway in the myotome (Munsterberg *et al*., 1995, Maroto *et al*., 1997) (Figure 1.2). At the same time, Wnt signalling proteins particularly Wnt-1, Wnt-3, and Wnt-4 produced from surface ectoderm and neural tube also induce the expression of skeletal muscle-specific genes MyoD and myosin heavy chain (MHC) in cells (Münsterberg *et al*., 1995). The induction of Myf5 and MyoD are required for specification and commitment of mesodermal somitic cells to the myogenic lineage to become myoblasts.

Besides positive regulatory signals, negative regulatory signals are present in the environment to inhibit and modulate the process of myogenesis. For example, growth factor such as bone morphogenetic proteins (BMP) is released from the lateral plate mesoderm to inhibit myogenesis. Concomitantly, the BMP antagonist Noggin is expressed within the dermomyotome to block BMP inhibitory activity. During the normal myogenic cell development, a low level of BMP signalling regulated by Noggin is necessary to control the ability of Pax3 cells to induce MyoD and Myf5 expression (Reshef *et al*., 1998). Also, in the dorsal lip of the dermamyotome, MyoD

family inhibitor (Mdfi) is expressed in low amounts to modulate Myf5 expression during the specification of the skeletal muscle lineage (Kraut *et al*., 1998). Hence, positive and negative regulatory factors are essential and act together in a tightly regulated framework to control and commit progenitor cells into muscle lineage.

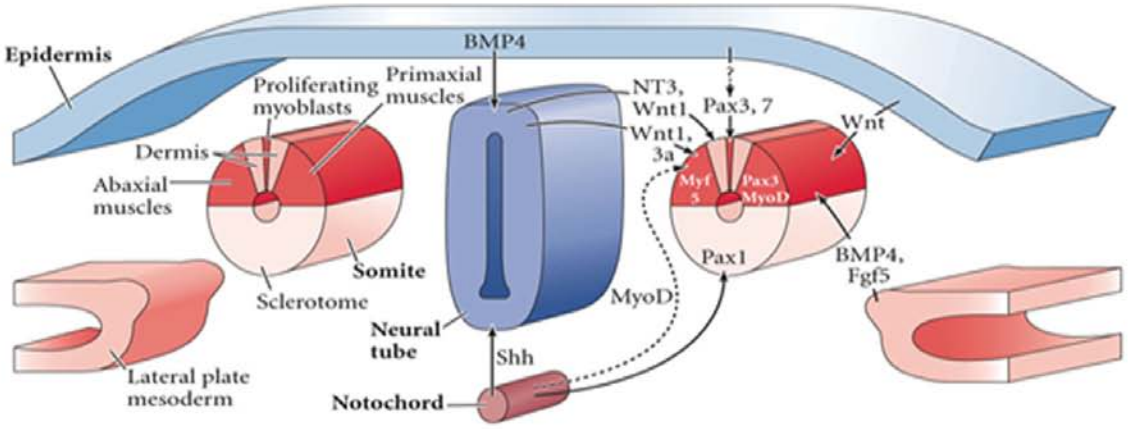

Figure 1.2. The myogenic regulatory network (Taken from Scott F. Gilbert, 2010).

1.1.2. The myogenic regulatory network in embryonic muscle development - differentiation of myoblasts into myotubes

While the commitment of progenitor cells to myoblast cell fate is regulated by microenvironmental proteins, the proliferation and differentiation of myoblasts into myotubes (which form the muscle fibers) are regulated by MRFs. MRFs such as MyoD and Myf5 are upregulated by Pax proteins. At the same time that these proteins are upregulated, many mitogenic growth factors are induced. Mitogenic factors such as fibroblast growth factors and hepatocyte growth factors are secreted by the mesoderm cells to induce MEK/ERK signalling pathway. These signalling pathways

are required for myoblast proliferation and migration during embryonic muscle development (Li *et al*., 2006). After several rounds of myoblast proliferation, MyoD and Myf5 upregulate the late MRFs such as myogenin and MRF4 which will in turn induce the expression of terminal differentiation genes such as troponin T, myosin heavy chain (MHC) and muscle creatine kinase (MCK). These muscle genes are required for terminal differentiation and fusion of myoblasts to form multinucleated myotubes.

Hence, the process of differentiation towards the formation of skeletal muscle involves activation of positive regulating factors such as MRFs, growth factors and environmental signals. In addition, negative regulating factors are present to act in a concerted manner with positive regulating factors to regulate the determination, proliferation and terminal differentiation of embryonic muscle progenitors into muscle fibers. It is also important to note that where negative regulating factors are present in excess, they can affect proper functioning of MRFs, leading to abnormal skeletal muscle development.

1.2. Adult skeletal muscle regeneration

In the late stages of muscle development, while some myoblasts fuse to form skeletal muscle fibers, others develop into muscle-specific stem cells known as satellite cells. These satellite cells reside beneath the basal lamina of adult muscle fibers, are mitotically quiescent until local environmental stimuli appear. They are responsible for postnatal muscle growth and regeneration of adult myofibers after an injury (Schultz *et al*., 1978). In response to stimuli such as muscle injury, exercise and electrical stimulation, satellite cells emerge from the basal lamina, become activated to proliferate and express myogenic genes required for skeletal muscle differentiation.

Eventually, these cells differentiate and form new myofibers which fuse to existing muscle fibers to regenerate and repair damaged skeletal muscles.

1.2.1. Adult skeletal muscle regeneration - activation of satellite cells

Satellite cells in their quiescent and unproliferative state do not express MyoD but express Pax7 which is required for the maintenance of adult satellite cells and generation of committed progenitors (Seale *et al*., 2000, Relaix *et al*., 2006, Kuang *et al*., 2006). In response to environmental stress or muscle injury, muscle satellite cells will be activated and move out of the basal lamina. At the same time, inflammation occurs with fibroblastic growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor-I (IGF-I), cytokines and non-muscle mononucleated cells including neutrophils and macrophages are attracted to the site of injury. Other proteins such as leukemia inhibitory factors, IL-6, IL-15 and TNF-alpha also aggregate at the site of muscle damage. Together, these growth factors and proteins induce the satellite cells to exit its quiescent state and proliferate as myoblasts. Eventually, cells proliferate with proliferating cell nuclear antigen (PCNA, a maker for cell proliferation) expressed to promote the expression of MyoD in myoblasts. Although, MyoD is constitutively expressed in proliferating myoblasts, it is kept in a transcriptionally inactive form. MyoD can only be activated to execute the muscle differentiation program, upon the depletion of the growth factors and cessation of myoblast proliferation (Lassar *et al*., 1994).

1.2.2. Adult skeletal muscle regeneration - terminal differentiation of myoblasts

After several rounds of proliferation when the growth factors are depleted, Pax3 and Pax7 are downregulated. MyoD is activated to stimulate terminal differentiation of

myoblasts by upregulating myogenin and MRF4 expression. At the same time, MyoD induces expression of CDK inhibitor p21, which binds and inhibits cyclin-CDK2 or cyclin-CDK4 complexes. p21 promotes myoblast withdrawal from the cell cycle, a process that is necessary for myoblasts to express muscle-related genes and differentiate into myotubes (Guo *et al*., 1995, Hawke *et al*., 2003). After myoblasts exit the cell cycle, myogenin and MRF4 induce expression of terminal differentiation genes such as troponin T and MHC which are necessary to drive cellular differentiation and fusion of myoblasts. Troponin T and MHC are the major components of the contractile apparatus in muscle fibers. In addition to troponin T and MHC, structural and contractile proteins are produced to form new myofibers repairing the damaged muscle fibers within 4-5 days (Kelly and Zacks, 1969, Bischoff, 1975, Konigsberg *et al*., 1975, Lipton and Schultz, 1979, Ontell and Kozeka, 1984, Megeney *et al*., 1996, Sabourin *et al*., 1999, Zammit *et al*., 2002, Relaix *et al*., 2005).

While a population of myoblasts undergoes terminal differentiation, a fraction of myoblasts maintain Pax7, exits cell cycle and relocate to the basal lamina. These cells maintain a viable muscle satellite cells reservoir (Baroffio *et al*., 1996, Olguin *et al*., 2004) so that they can continue to respond efficiently to repeated muscle injury (Luz *et al*., 2002, Sadeh *et al*., 1985).

Similar to embryonic muscle development, myogenesis in adult muscle regeneration is also orchestrated through a transcriptional regulatory network controlled by MRFs. With coordinated transcriptional activities, muscle progenitors are activated to proliferate and subsequently undergo terminal differentiation. In the adult muscle regeneration, MRFs-associated negative regulatory proteins regulate muscle gene expression. Therefore, an imbalance between MRF and its negative regulatory

proteins in association can cause abnormal transcriptional activity and inefficient muscle regeneration during muscle injury.

1.3. Transcriptional control of myogenic differentiation

Skeletal muscle differentiation is controlled by two groups of myogenic transcription factors, the MRFs and the myocyte enhancer binding factor 2 (MEF2) proteins. MRFs such as MyoD, Myf5, Myogenin and Myf-6/MRF4/herculin are important basic-helix-loop-helix (bHLH) positive transcriptional regulators critical for controlling the fate, migration, proliferation, differentiation, regeneration and survival of muscle cells. Generally, there are two separate processes in myogenesis: myoblast proliferation and differentiation. During myoblast proliferation, in the presence of growth factors, MyoD, Myf5 and E-proteins are expressed but both MyoD and Myf5 remain inactive. Upon deprivation of growth factors, muscle determination gene MyoD and Myf5 are activated to initiate cell differentiation. MyoD and Myf5 heterodimerize with ubiquitously expressed E-proteins (E12/E47/HEB) through their HLH domains. The resulting myogenic MyoD-E / Myf5-E heterodimers bind to E-box sites (CANNTG consensus sequence) present in promoters of many muscle-specific genes such as myogenin and MRF4 and transcriptionally induce gene expression (Lassar and Munsterberg, 1994, Arnold and Winter, 1998). Subsequently, expression of muscle differentiation genes myogenin and MRF4 trigger muscle terminal differentiation and stimulate synthesis of muscle-specific structural and contractile proteins such as MHC, α-actin, muscle creatine kinase, fast muscle-type and slow muscle-type of troponin T required for myofiber formation (Yutzey *et al.*, 1990, Muscat *et al.*, 1992, Li and Capetanaki, 1993, Ziober and Kramer, 1996, figure 1.3). However, in addition to MyoD and Myf5, studies have shown that MRF4, being normally described as a

differentiation gene, was also found to function a determination gene *in vivo* (Kassar-Duchossoy *et al*., 2004).

The onset of terminal differentiation is preceded by cells exiting the mitotic cell cycle. Hence, besides inducing muscle gene expression for terminal differentiation, MyoD also binds to E-box sites and activates expression of the cell-cycle inhibitor p21 which mediates cell cycle exit (Figure 1.3) (Guo *et al*., 1995, Hawke *et al*., 2003). Induction of p21 leads to increased inhibition of cyclinD1-CDK4 and cyclinE-CDK2 activities which results in dephosphorylated retinoblastoma (Rb) binding to E2F and blocking transcription of cell cycle genes. Cell cycle exit is necessary to facilitate the expression of late skeletal muscle differentiation genes required for the myoblast differentiation (Huh *et al*., 2004).

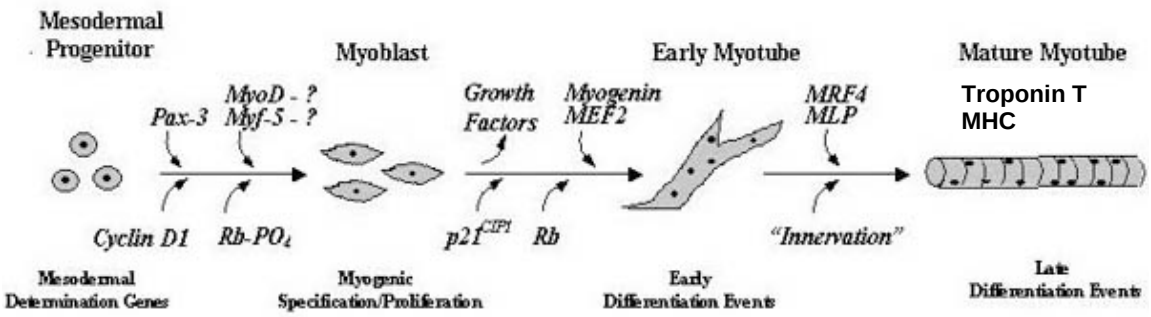

Figure 1.3. The myogenic differentiation pathway (Taken from the Konieczny lab website- www.bio.purdue.edu/people/faculty/konieczny/lab/overview.htm).

During myogenic differentiation, MRFs also up-regulates MEF2 which binds MADS-box promoter sites and activates transcription of muscle structural genes. MEF2 factors are different from MRFs as they do not possess myogenic activity on their

own. However, MEF2 can enhance MRFs activity in the regulation of muscle gene expression (Molkentien *et al.,* 1996, Black and Olson, 1998).

Besides the example of MRFs and MEF2 activating transcription in a co-operative manner, it has been documented that most transcription factors often work in concert with transcription co-factors in modulating the activation of myogenic gene expression. The initiation of myogenesis by these MRFs is also accompanied by combined histone acetylation/deacetylation, histone methylation/demethylation of a particular histone lysine at regulatory regions or post-translational modifications of the transcription factors by phosphorylation or sumoylation (Eusebio *et al*., 2009).

1.3.1. Deficiency of MRFs in muscle development

Many *in vivo* knockout studies determined that MRFs play important functional roles during muscle development. Although mice lacking either the MyoD or Myf5 gene develop normal skeletal muscle, knockout of both MyoD and Myf5 inhibited myoblast formation and reduced muscle masses (Rudnicki *et al.*, 1993). Mutation of Myogenin gene in mice resulted in reduced myofibers, fractures in ribs and death at birth (Hasty *et al.,* 1993, Nabeshima *et al.,* 1993). Mice lacking MRF4 displayed no overt defect in muscle development with only slight reduction of muscle specific genes (Olson *et al*., 1996). However, mice with double knockout of MyoD and MRF4, were phenotypically similar to myogenin knockout mice. In addition, these MyoD and MRF4 double mutants mice exhibited lethal deficiency of myofiber formation albeit the presence of the myogenin expression (Rawls *et al*., 1998).

As described earlier, Pax3 and Pax7 function upstream of MRFs. Together, they are required for the determination and terminal differentiation of embryonic muscle progenitors. Some studies demonstrated that a homozygous Pax3 gene mutation

resulted in muscle weakness where myogenic progenitors failed to migrate from somites into the growing limbs and the developing tongue (Bober *et al*., 1994, Goulding *et al*., 1994, Tremblay *et al*., 1998). Similarly, other studies showed that Pax7 knockout newborn mouse has impaired regenerative capacity resulting in reduced muscle mass and shorter survival time (Mansouri *et al*., 1996, Oustanina *et al*., 2004). It has been shown that a double knockout of Pax3 and Pax7 in mice resulted in embryonic lethality and inhibition of myogenesis. In addition, improper trunk muscle development with inhibited MyoD expression was reported in Pax3 and Myf5 double mutant mice (Tajbakhsh *et al*., 1997). Hence, MRFs are critical muscle regulators in muscle development as any disruption in their expressions or activities can hinder proper muscle differentiation and lead to muscle disorder.

1.4. Chromatin modifications in myogenesis

Gene expression is regulated by transcription factors. However, gene expression is also epigenetically modulated, by post-translational modification of histone tails. Chromatin found in a repressive or transcriptionally inactive state can be switched to an in active state by several chromatin modifiers and remodeling proteins or vice versa. Chromatin modifiers also known as transcriptional co-factors do not bind directly to DNA but bind to the regulatory transcription factors instead to modulate gene transcription activity. When bound to the transcription factors, the transcription co-factors alter the chromatin structure to obstruct or enhance RNA polymerase from transcribing genes. Alteration of the chromatin structure takes place through epigenetic chemical modifications of the N-terminal tail regions of the core histones H2A, H2B, H3 and H4 (which are tightly bound to the DNA) by acetylation, methylation, phosphorylation, sumoylation and/or ubiquitylation (Kouzarides, 2007).

Similarly, in the myogenic regulatory network, while MRFs govern the ordered and specific expression of muscle genes required for terminal differentiation of myocytes into myofibers, their transcriptional activity can be altered by their associated transcriptional co-factors. There exist numerous transcriptional co-factors modifying the architecture of the chromatin at specific region and activate/repress the transcription of muscle differentiation gene.

1.4.1. Chromatin modifications by histone deacetylases

During the regulation of skeletal muscle gene expression, transcription factors MRFs and MEF2 do not work alone but are associated with histone acetylases (HATs) and histone deacetylases (HDACs) to activate or repress the myogenic genes (Dressel *et al*., 2001, Terranova *et al*., 2005). HATs and HDACs are enzymes that acetylate/deacetylate lysines on histones which can affect DNA-binding properties and gene expression. Histone lysine acetylation neutralizes the positive charge on the histones and decreases the histones ability to bind DNA. This permits DNA accessibility to the transcription factors and thus allows activation of gene transcription. Histone lysine deacetylation, on the other hand, increases histones affinity binding to the DNA and leads to chromatin condensation. A condensed chromatin structure prevents access of transcription factors to DNA and thus represses gene transcription. There are three classes of HDACs which are classified into three categories based on their homology to yeast proteins Rpd3p (class I), Hda1p (class II), and Sir2p (class III) (De Ruijter *et al*., 2003, North and Verdin, 2004). The deacetylase activity of class III HDAC is dependent on co-factor NAD and the activity of class I and II HDACs can be inhibited by Trichostatin A (TSA). These HDACs are implicated in the regulation of skeletal myogenesis where they can

physically associate with and restrain MyoD and MEF2 in a transcriptionally inactive state (Dressel *et al*., 2001, Terranova *et al*., 2005).

In undifferentiated myoblasts, muscle differentiation genes are epigenetically marked for repression. MyoD is a dominant positive myogenic factor which is capable of binding to and initiating the expression of skeletal muscle genes (Salvatori *et al*., 1995). Tapscott's group showed that although MyoD is bound to muscle promoters in both differentiated myotubes and undifferentiated myoblasts. MyoD does not transcriptionally activate genes in myoblasts (Cao *et al*., 2010). In undifferentiated myoblasts, MyoD is expressed but kept in a deacetylated and transcriptionally inactive form by several epigenetic mechanisms (Mal *et al*., 2001). MyoD binds to HDAC1, via its bHLH domain and deacetylates histones on MyoD-target sites on promoters, resulting in a transcriptionally repressive chromatin state (Fulco *et al*., 2003, Mal and Harter, 2003, Mal *et al*., 2001). HDACs have also been shown to have inhibitory roles in myogenesis. Overexpression of HDAC1 inhibits myoblast differentiation by deacetylating histones on promoters of late muscle genes such as MCK and MHC (Puri *et al*., 2001, Mal *et al*., 2001). In addition to deacetylating histones, HDAC1 deacetylates MyoD and kept it in a transcriptionally inactive form in undifferentiated myoblasts (Mal *et al*., 2001).

With the onset of differentiation, myoblasts irreversibly withdraw from the cell cycle. Hence, in differentiating myoblasts, cell cycle genes are kept in a transcriptionally repressive state (Caretti *et al*., 2004, Zhang *et al*., 2002). The retinoblastoma protein (Rb) plays important roles in controlling cell cycle gene expression during myoblast differentiation (Huh *et al*., 2004). In undifferentiated myoblasts, HDAC1 is associated with MyoD but during the induction of myoblast differentiation, hypophosphorylated Rb competes with and replaces MyoD from the inhibitory MyoD-HDAC1 complex.

Thus, Rb-HDAC1 complex represses E2F target genes and silences S-phase genes whereas, MyoD is intact and activated to initiate myogenic transcription (Puri *et al.*, 2001, Blais and Dynlacht, 2007). Subsequently, as the myoblasts begin to differentiate, acetyltransferases p300/CBP associated factor (P/CAF) and p300 form a multimer complex with the dissociated MyoD which will bind to the myogenin promoter and activate expression of muscle genes (Puri *et al*., 1997a, Puri *et al*., 1997b, Sartorelli *et al*., 1997, Sartorelli *et al*., 1999). While P/CAF acetylates MyoD at lysine K99, K102 and K104 required for MyoD transcriptional activity, p300 acetylates histones H3 and H4 on the myogenin promoter (Puri *et al*., 1997, Sartorelli *et al*., 1999, Dilworth *et al*., 2004).

However, these activities of co-activators can also be inhibited by transcriptional co-repressors. The co-repressor HDAC3 associates with the acetyltransferases p300 and P/CAF to block myogenesis (Gregoire *et al*., 2006). In addition, HDAC4 and HDAC5 can interact and block transcription factor MEF2 activity and subsequently repress transcription of both early and late muscle specific genes (Miska *et al*., 1999, Lu *et al*., 2000, McKinsey *et al*., 2001, Wang *et al*., 1999, Lemercier *et al*., 2000). However, such myogenic repression mediated by the transcriptional co-repressors can be blocked by HDAC inhibitors.

A class III HDAC-Sirt1/Sir2 forms a complex with MyoD and P/CAF and inhibited myogenesis. When NAD+/NADH+ ratio increases, Sirt1 blocks MyoD activity and induces histone deacetylation at muscle promoter, thus inhibiting differentiation. On the other hand, as the NAD+/NADH+ ratio decreases, Sirt1 activity reduces, allowing P/CAF to acetylate histones and MyoD (Fulco *et al*., 2003).

1.4.2. Chromatin modifications by methyltransferases

Methyltransferases methylate lysine and arginine residues within histones or non-histone proteins. Lysine residues can be mono-, di- or tri-methylated by several methyltransferases such as Suppressor of variegation 3-9 homologue 1/2 (Suv39h1/2), G9a-like protein (EHMT1/GLP), Polycomb group protein Enhancer of zeste 2 (Ezh2), SETDB1 / ESET and EHMT2/G9a. These methyltransferases can act either as co-activators or co-repressors with the DNA binding transcription factors to activate or repress gene transcription, respectively (Jenuwein *et al*., 2001 Zhange and Reinberg, 2001). For example, methylation of histone 3 lysine 9 (H3K9) is associated with inactive genes and tri-methylation of H3K9 is associated with pericentromeric heterochromatin and transcriptional repression. On the other hand, methylation of H3K4 and H3K17 have been generally associated with active genes and di-methylation of H3K9 is associated with repression of transcription genes in euchromatin (Lee *et al*., 2005).

In myoblasts, high levels of H3K9 methylation on the myogenin promoters, is associated with gene repression (Zhang *et al.*, 2002, Mal and Harter, 2003). The histone methyltransferase Suv39h1 is recruited by MyoD to modify chromatin structure and block muscle gene expression. Suv39h1 tri-methylates H3K9 via its histone methyltransferase activity and inhibits myogenic differentiation (Mal, 2006). On the other hand, it has been suggested that Suv39h1 methylation activity is required during myogenic cell differentiation to repress cell-cycle genes. Their reports showed that reduced level of methyltransferase Suv39h1 was unable to express muscle genes and differentiate into myotubes (Ait-Si-Ali *et al*., 2004).

In addition, the methyltransferase polycomb Ezh2 has been shown to be recruited by a non-muscle transcriptional regulator YY1 to add repressive chromatin marks on MCK

and MHCIIb genes promoters. Ezh2 di- and tri-methylates H3K27 through its HMKT activity thereby represses muscle gene expression (Caretti *et al.*, 2004).

Other methyltransferases such as Carm1/Prmt4 interacts with MEF2 proteins and di-methylates H3R17 at MCK promoter, whereas Prmt5 associates with MyoD and di-methylates H3R8 at both myogenin and MCK, dystrophin promoters (Chen *et al*., 2002, Dacwag *et al*., 2009). Carm1/Prmt4 binding at late- myogenic promoters promotes binding of the Brg1 ATP-dependent chromatin-remodeling enzyme and subsequent chromatin remodeling at the promoter (Chen *et al*., 2002, Dacwag *et al*., 2009). Hence, chromatin modification by methyltransferases is involved in the silencing myogenic gene expression and maintaining myoblasts in an undifferentiated state.

1.4.3. Chromatin modifications by kinases and phosphatases

It has been documented that kinase and phosphatase have roles in the regulation of myogenic differentiation. Studies have shown that kinases cdk1 and cdk2 phosphorylate MyoD and restrict its activity. Phosphorylated MyoD is targeted for ubiquitination and degradation (Song *et al*., 1998). MyoD is highly phosphorylated by cdk1 and cdk2 in proliferating myoblasts and is dephosphorylated in differentiating myoblasts (Kitzmann *et al*., 1999).

Protein kinase C (PKC), activated in the presence of fibroblast growth factor, phosphorylates myogenin and blocks its DNA binding activity (Li *et al*., 2005). Protein kinase A (PKA), on the other hand, has been shown to phosphorylate and inhibit both Myf5 and MyoD activities (Winter *et al*., 1993). Similarly, cyclin-dependent kinase 4 (cdk4) binds directly to MyoD and disrupts its DNA binding and its transcriptional activity (Zhang *et al*., 1999a).

Other studies have indicated that mitogen-activated protein kinase p38-α is required to phosphorylate E47 at Serine140 to promote heterodimerization of E47/MyoD and transcription of muscle genes. The data show that when E47 is unphosphorylated, it fails to bind MyoD. Thus, the transcription factor MyoD alone cannot bind to regulatory sites and initiate transcription of muscle genes (Luis *et al*., 2005). Conversely, the isoform p38-γ phosphorylates Serine199 and Serine200 on the carboxy terminus of MyoD which enhances its interaction with transcriptional repressor histone methyltransferase Suv39h1 to inhibit myogenesis (Lassar, 2009). Hence, kinases and phosphatases have both activating and inhibitory functions in regulating MRF activity.

Other chromatin modifiers include the ATPase-dependent SWI/SNF remodeling complexes which are recruited to muscle promoters to activate muscle differentiation (Simone *et al*., 2004, Albini and Puri 2010). During differentiation, p38 alpha/beta kinases are activated to promote SWI/SNF recruitment onto the chromatin of muscle genes to induce muscle differentiation.

Thus, MRFs and epigenetic regulators play important roles in modulating the activity of MRFs and myogenic differentiation. There is a network of functional interactions between transcription factors and chromatin-modifying complexes that regulate the muscle gene-specific chromatin conformation during the transition of myoblasts to myotubes.

1.5. Regulation of myogenesis by repressors

A number of proteins which negatively regulate myogenesis have been identified. For example, bFGF (basic fibroblast growth factor), TGF-β (transforming growth factor), Myostatin, and Notch signalling have been shown to inhibit proliferation of muscle

stem cell and to block recruitment and fusion of myoblasts into myofibers (Kopan *et al*., 1994, Nofziger *et al*., 1999, Langley *et al*., 2002, Yoshiko *et al*., 2002). Activation of Notch1 leads to satellite cell proliferation but prevents differentiation (Conboy and Rando, 2002). Various other oncogenes such as c-myc, N-ras, and Ha-ras also inhibit muscle differentiation *in* v*itro* (Olson *et al*., 1987). These proteins or growth-promoting factors negatively regulate the transcriptional activity of MRFs in myogenic differentiation.

Besides signalling proteins, HLH transcriptional repressors have been shown to block muscle gene expression. MRFs bind to the E-box regulatory region to activate transcription of muscle genes, but at the same time, negative regulators can compete for the same regulatory region to block MRFs from binding and triggering muscle gene transcription. Also, the repressors can bind directly to the MRF to suppress its transcriptional activity (Lemercier *et al*., 1998, Rawls *et al*., 1998, Azmi *et al*., 2003). One example is the inhibitor of DNA binding/differentiation (Id) family protein which is a HLH protein but lacks a basic DNA-binding domain. Id, through its HLH domain, binds to MRFs and prevents it from binding to the E-box (Benezra *et al*., 1990). It also heterodimerize with E-proteins and prevents the formation of functional MRF-E-protein heterodimer to bind E-box, leading to a repression of myogenic genes and inhibited myoblasts differentiation. Other bHLH transcriptional repressors of muscle gene include Twist, Mist1, MyoR (myogenic repressor), Hes1 (Hairy and Enhancer of Split), Hey1 (Hairy/ enhancer-of-split-related with YRPW motif 1), Hesr (hairy and enhancer of split related HESR family) and Sharp-1 (Enhancer of split and Hairy-related protein 1). Twist interacts with the basic domain of MyoD and represses MyoD transactivation. Similar to Id, Twist sequesters E-proteins, inhibits MEF2 activation and blocks P/CAF and p300 histone acetylase activity (Spicer *et al.*, 1996).

In myogenesis, both the transcriptional co-activators p300 and P/CAF are necessary for MyoD to activate skeletal muscle program. Akin to the other repressor's mechanism in attenuating MyoD transcriptional activity, Mist1 heterodimerizes with E-protein, occupies E-box sites and blocks muscle gene expression or heterodimerizes with MyoD resulting in inactive MyoD (Lemercier *et al*., 1998). Other inactive forms of MyoD heterodimers include Hes1/MyoD (Sasai *et al*., 1992) and Sharp-1/MyoD (Azmi *et al*., 2004), negatively regulate myogenesis. These repressors are also known to block myogenesis, however, the details regarding the molecular mechanism, biological pathway of the repressors-mediated inhibition of MRFs remains to be determined.

Among these repressors, our laboratory is interested and focused on the role of Sharp-1 in regulating cellular proliferation and differentiation. Sharp-1 has been identified as a transcriptional repressor in both myogenic differentiation and adipocyte differentiation (Azmi *et al*., 2004, Gulbagci *et al*., 2009). The molecular mechanisms underlying Sharp-1-mediated repression of myoblast differentiation remain unclear (Azmi *et al*., 2004).

1.6. The transcriptional repressor Sharp-1

Mus musculus Sharp-1 (Enhancer-of-Split and Hairy-related protein 1), also known as *homo sapiens* DEC2 (differentiated embryo chondrocyte protein 2), BHLHE41 or bHLHB3, is a 410 amino acid transcription factor which is involved in the control of proliferation and differentiation of several cell types including epithelial cells, nerve cells and fibroblasts (Sato *et al*., 2008, Liu *et al*., 2009, Cho *et al*., 2009, Garriga-Canut *et al*., 2001, Gulbagci *et al*., 2008, Azmi *et al*., 2004).

The mouse SHARP-1 gene maps to chromosome 6 G2-G3 and the human homologue DEC2 gene maps to chromosome 12p11.23-p12.1 (Fujimoto *et al.,* 2001). Sharp-1 contains a bHLH domain at the N-terminus, an orange domain and an alanine / glycine-rich domain at the C-terminus. Its basic region contains an Arg 13 residue which is important for DNA-binding to the consensus sequence CACGTG. Its adjacent HLH region mediates homo- or hetero-dimerization with other HLH proteins. Phylogenetics analyses revealed that Sharp-1 belongs to the bHLH transcription factors (Hey, Hes, Stra13/DEC1) family (figure 1.4) that regulate many physiological processes such as cellular differentiation, cell cycle arrest and apoptosis (Sun *et al*., 2007). Sequence alignment shows that it shares highest sequence homology (89%, 366 out of 410 amino acid conserved) to Sharp-2/Stra13 (stimulated by retinoic acid 13 homolog) /DEC1 with 95% amino acid identity in their bHLH domain. The bHLH region of Sharp-1 is structurally similar (40%) to that of Hes and Hey proteins.

Hes/Hey proteins, through their WRPW or YRPW domain, recruit co-repressor Groucho to repress transcription and suppress neurogenesis. Although Sharp-1 belongs to the bHLH family, it represses gene transcription differently from other members of bHLH transcription factors as its C-terminus lacks the WRPW or YRPW domain (Sun *et al*., 2007).

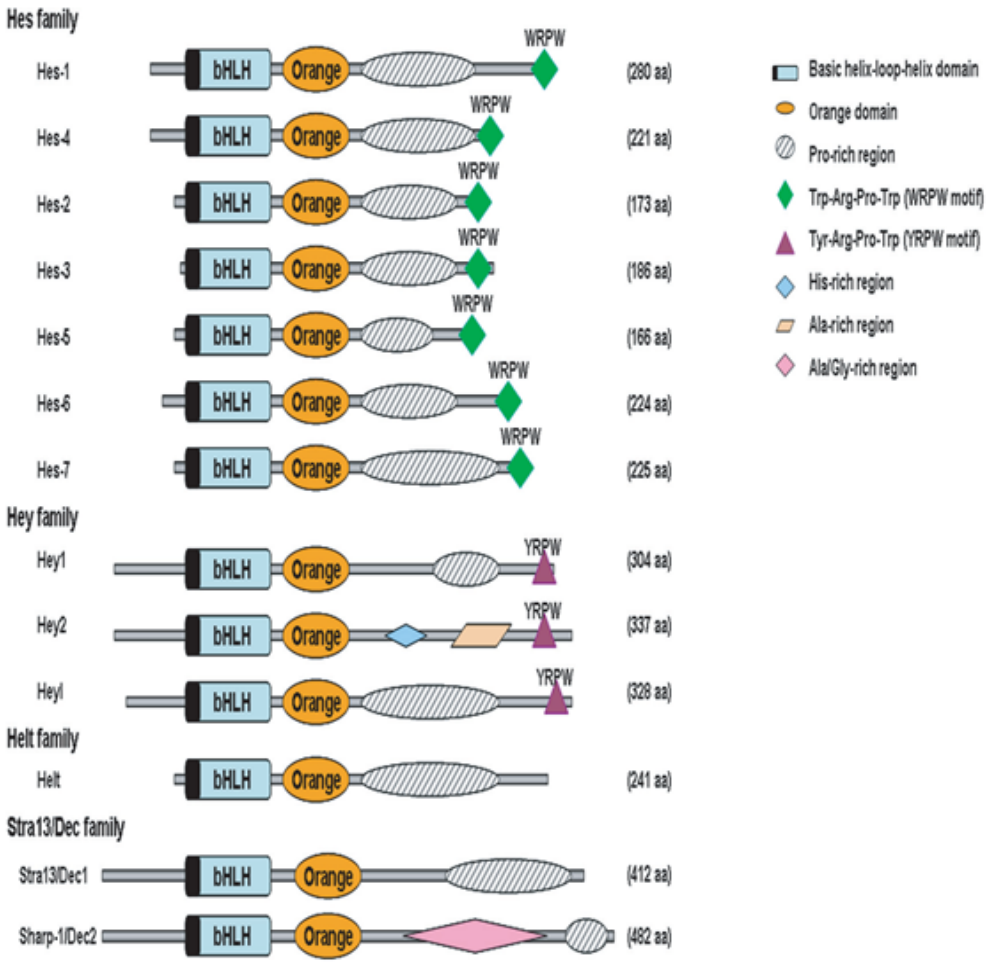

Figure 1.4. The domain structure of Sharp-1 (Taken from Sun *et al.*, 2007).

Sharp-1 expression is regulated by various extracellular stimuli such as growth factors, serum starvation, hypoxia and cytokines (Yamada and Miyamoto, 2005). Sharp-1, is expressed in various tissues, and plays an important role in multiple signalling pathways that impact many biological processes including development, cell differentiation, cell growth, cell death, circadian rhythms. Its mRNA is expressed at high levels in the heart, skeletal muscle, and brain and at low levels in the lung, placenta, pancreas and kidney and in both embryonic and adult tissues (Shen *et al.*,

1997, Fujimoto *et al*., 2001, Azmi *et al*., 2002). Unlike other bHLH protein, Sharp-1 is expressed in post-natal neurons but not in neuronal progenitor cells or early differentiating neurons, suggesting that it has a role in terminal neuronal differentiation (Rossner *et al*., 1997).

1.6.1. Transcriptional repression by Sharp-1

In most cells, Sharp-1 mainly functions as a transcriptional repressor which silences gene expression. Several mechanisms are involved in Sharp-1-dependent transcriptional repression. The first mechanism is dependent on binding of Sharp-1 homodimers to class B E-box elements (CACGTG), resulting in repression of transcription. For instance, Sharp-1 binds directly to E box elements in the Stra13 promoter and inhibits its expression. Stra13 shares the highest sequence homology with Sharp-1, functions as a transcriptional repressor and is involved in the control of differentiation of several cell lineages during mouse development. In addition, Sharp-1 also sequesters the transcription factor Sp1 and impedes its transcription activity on Stra13 promoter (Kawamoto *et al*., 2004, Sun and Taneja, 2000, Azmi *et al*., 2003). A second mechanism is by interaction with other transcription factors blocking their activity and resulting in repression of gene expression (Azmi *et al*., 2003, Yamada and Miyamoto 2005). Previous studies from our laboratory have shown that Sharp-1 negatively regulates adipose cell differentiation. Sharp-1 attenuates the transcriptional activity of both CCAAT/enhancer binding protein α and β (C/EBPα and C/EBPβ) required for the development of white adipose tissues. This reduced transcriptional activity leads to inhibition of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) genes expression and adipocytic differentiation (Gulbagci *et al*., 2009). Apart from inhibiting adipogenesis, Sharp-1 negatively regulates genes required for

myogenic differentiation. The inhibitory activity of Sharp-1 occurs through sequesting of MyoD and E-protein, thus resulting in a block of MyoD transcriptional activity (Azmi *et al.*, 2004).

Sharp-1 has been reported to recruit HDAC1 through its C-terminus that may be important for repression of specific target genes (Garriga-Canut *et al*., 2001). For instance, Sharp-1 represses muscarinic receptor gene and its repressive effect can be overcome with a histone deacetylase inhibitor Trichostatin A (Garriga-Canut *et al*., 2001). Similarly DEC2 (Sharp-1) represses transactivation of retinoid X receptor (RXR) and the retinoid X receptor-liver X receptor (RXR-LXR) heterodimers, in a HDAC-dependent mechanism. Both RXR and LXR are transcription factors required for cell growth and differentiation in sebocyte and myeloid cells (Cho *et al*., 2009, Kim *et al*., 2000).

Sharp-1/DEC2 plays a role in the regulation of circadian rhythms. Brain and muscle Arnt-like protein-1 (BMAL1) is a transcription factor known to regulate circadian rhythm. Both Sharp-1/DEC2 and Stra13/DEC1 repress the transcriptional activity of BMAL1 through direct protein-protein interaction with BMAL1 or competition for binding to the E-box elements (Hamaguchi *et al*., 2004, Honma *et al*., 2002, Kawamoto *et al*., 2004, Sato *et al*., 2004)

It has also been shown that Sharp-1 functions as a transcriptional repressor in the paracrine regulation of ductal growth and differentiation. Sharp-1 binds and hinders hypoxia-inducible factor, a transcription factor from binding to the promoter, resulting in downregulation of vascular endothelial growth factor (VEGF) expression (Sato *et al*., 2008).

Surprisingly, besides being a repressor, a recent study demonstrated that Sharp-1 can perform dual functions as a repressor or activator of clock gene expression depending

on the nature of E-box binding complexes in different tissues. Sharp-1 serves as a repressor of E-box-mediated transcription in the cortex and liver. On the contrary, through rapid delays of the light-dark cycle (experimental jet lag) and light-pulse experiments, it was shown that Sharp-1 induces expression of light-responsive genes c-Fos, c-Jun and Per1 in the suprachiasmatic nucleus (Rossner *et al*., 2008).

1.6.2. Role of Sharp-1 in myogenesis

Sharp-1 mRNA is expressed in proliferating C2C12 myoblast cells. Its expression declines during myogenic differentiation which suggests that Sharp-1 may have a role in keeping myoblasts in an undifferentiated proliferative state. Consistent with these studies, overexpression of Sharp-1 in C2C12 myoblast cells has been shown to inhibit myoblast differentiation into myotubes. Moreover, using myogenic conversion assays in C3H10T½ mouse fibroblast cells, Sharp-1 was found to inhibit MyoD-dependent myogenic differentiation and expression of myogenin, MEF2C and myosin heavy chain during differentiation (Azmi *et al*., 2004).

This inhibitory effect of Sharp-1 is in part due to inhibition of the transcriptional activity of E protein-MyoD complexes. Sharp-1 heterodimerizes with transcription factors MyoD and E-proteins, through their HLH domain and prevents the formation of MyoD/E47 dimers and thus blocks myogenic differentiation (Garriga-Canut *et al*., 2001, Azmi *et al*., 2004, Fujimoto *et al*., 2007). Importantly, even though Sharp-1 represses transcription through its bHLH domain, a tethered MyoD-E47 dimer is still unable to completely overcome Sharp-1-mediated repression. This suggests that Sharp-1 has more than one mechanism in inhibiting myogenic differentiation (Azmi *et al*., 2004). Hence, Sharp-1 has an inhibitory role in myogenic differentiation and its bHLH domain is not the only mechanism in blocking muscle gene expression. In

the neurons, Sharp-1 abrogates gene expression in a HDAC-dependant manner (Garriga-Canut *et al*., 2001). Interestingly and in contrast, in muscle cells, HDAC1 is not involved in Sharp-1-mediated repression of MyoD activity (Fujimoto *et al*., 2007). Previous studies from our laboratory have shown that Sharp-1 recruits transcriptional co-factors HDAC1 and G9a onto C/EBPα and PPARγ promoters during the inhibition of adipocyte differentiation (Gulbagci *et al*., 2009). This raised the possibility that Sharp-1 may recruit G9a to regulate muscle gene expression. This study therefore examines the role of methyltransferase G9a and its recruitment by Sharp-1 in myogenesis.

1.7. The methyltransferase G9a

The methyltransferase G9a, also known as Euchromatic histone lysine N-methyltransferase 2 (Ehmt2), Bat8, KMT1C and NG36, belongs to the histone-lysine methyltransferase family which includes Suv39h1, SET9 and Ezh2. The *Homo sapiens* G9a is located on chromosome 6 p21.31 (Brown *et al*., 2001). There are two isoforms, a long isoform with 1210 amino acids and a short isoform with 1001 amino acids. The *Mus musculus* G9a is located on chromosome 17. The long isoform consists of 1263 amino acids and the short isoform consists of 1172 amino acids. Ubiquitously expressed, G9a has been suggested to have 2 nuclear localisation signals in the Gly-Arg rich repeats and is localised exclusively in the nucleus. The carboxyl terminus of G9a consists of 6 ankyrin repeats that serve to interact with other proteins (Davis *et al*., 1991) and a conserved catalytic SET domain (about 130 amino acids) flanked by 2 cysteine-rich regions. The amino end of G9a has very little similarity with any conserved protein domain (Figure 1.5). It is a histone lysine methyltransferase (HKMT) enzyme that methylates specific lysine residues within the

histone tails. As described earlier, methylation of lysine on histones is usually associated with transcriptional repression. G9a preferentially mono- or di-methylates H3K9 and less efficiently H3K27. G9a recognizes Arg-Lys (RK) sequence and that its activity can be inhibited by methylation of the arginine residue (Rathert *et al*., 2008).

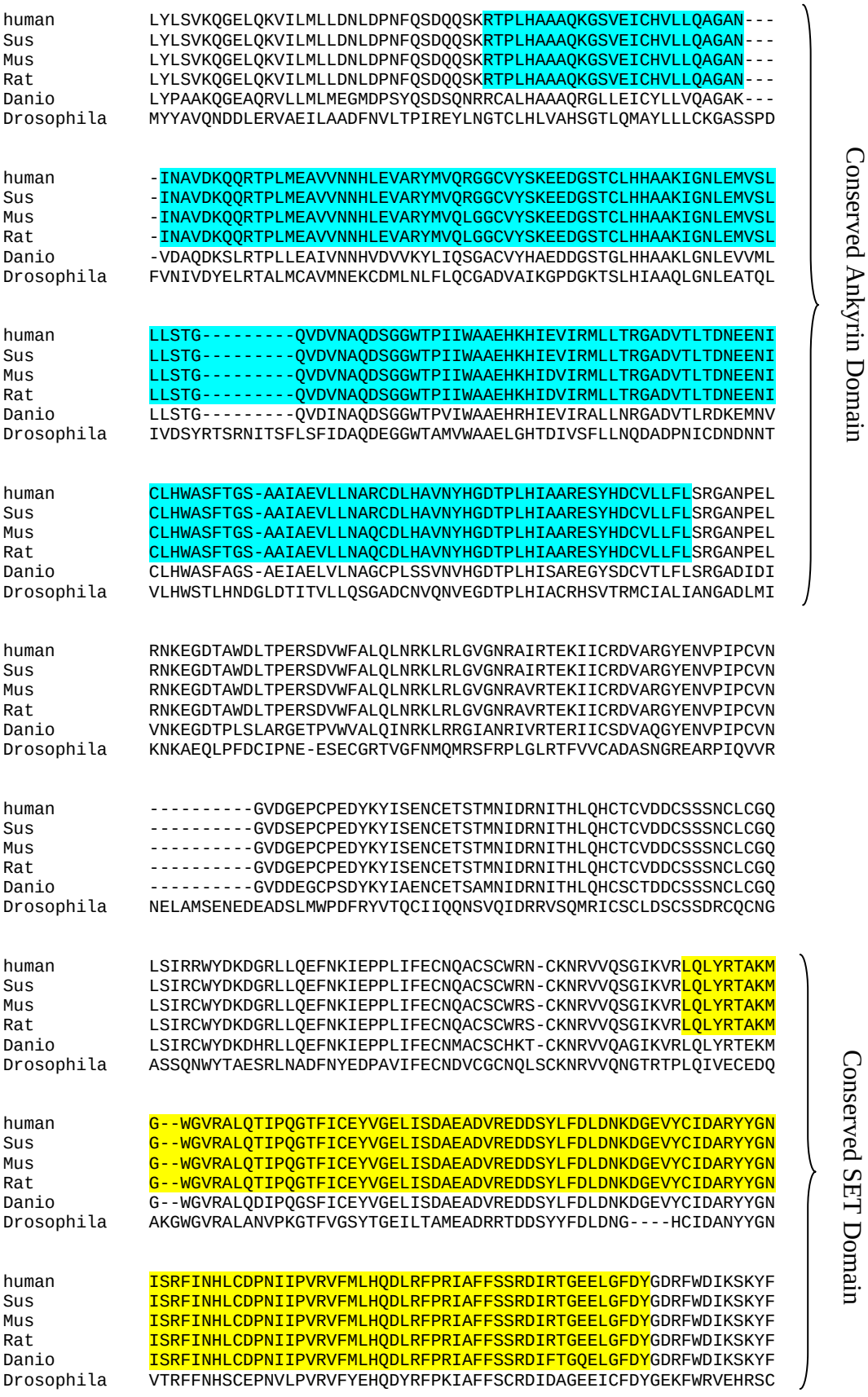

Figure 1.5. Protein sequence alignment of G9a in different species

G9a-mediated methylation is not restricted to histones. G9a has been shown to be capable of methylating non-histone proteins at the consensus Argine-Lysine (RK) amino acid such as CDYL1 (chromodomain Y-like protein), WIZ (widely interspaced zinc finger motifs protein), C/EBP, Reptin, ACINUS, CENP-A, CENP-B, CENP-C, TRF1 TRF2 and p53 and itself (Ueda *et al*., 2006, Chin *et al*., 2007, Rathert *et al*., 2008, Huang *et al*., 2010, Sampath *et al*., 2007, Pless *et al*., 2008, Lee *et al*., 2010) (Figure 1.6).

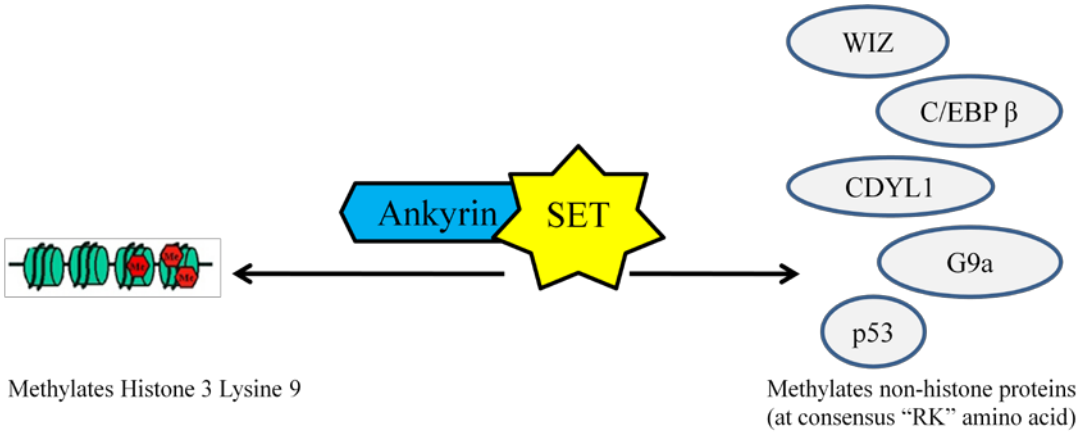

Figure 1.6. G9a methylates non-histone proteins. (Modified from Collins *et al*., 2010).

Other methyltransferases such as Ezh2 and Suv39h1 have been shown to inhibit myogenic differentiation. During the inhibition of myogenic differentiation, overexpression of Ezh2 increased H3K27 di- and tri-methylation levels at the MHC (late muscle marker) promoter (Caretti *et al*., 2004) whereas overexpression of Suv39h1 increased H3K9 tri-methylation level at the myogenin promoter (Mal *et al*., 2006). An increase in histone methylation is associated with repression in the gene expression (Jenuwein and Allis, 2001, Li *et al*., 2007).

G9a is a major H3K9 methyltransferase for euchromatin and is responsible for the transcriptional repression of many euchromatic genes (Gyory *et al*., 2004, Nishio and Walsh, 2004, Roopra *et al*., 2004, Duan *et al*., 2005, Tachibana *et al*., 2002, Kim *et al*., 2008).

1.7.1. Functions of G9a

The methyltransferase G9a is important in the methylation of euchromatic H3K9 and plays an important role in cellular differentiation, development and cell-cycle progression. The knockout of G9a in mice results in a decrease in global H3K9me1 and H3K9me2 levels. Moreover, mouse embryos deficient of G9a suffers from severe growth retardation and early lethality (Tachibana *et al*., 2002)

Previous studies found that methyltransferase G9a functions as a co-repressor which is recruited to several specific genes by associating with transcriptional repressors and co-repressors such as CDP/cut, Blimp-1/PRDI-BF1, and REST/NRSF.

A report showed that in many diverse cellular and viral genes, a transcriptional factor, CCAAT displacement protein/cut homolog (CDP/cut) involves G9a in regulating cellular processes, including differentiation, development, and proliferation. CDP/cut functions as a transcriptional repressor, recruits histone lysine methyltransferase G9a activity to repress transcription of p21 and inhibit cell cycle exit (Nishio and Walsh, 2004). Another similar report showed that UHRF1 recruits G9a to inhibit p21 promoter activity in regulating cellular proliferation (Kim *et al*., 2008). Similarly, it has been shown that the transcriptional repressor neuron restrictive silencing factor (NRSF/REST) can also recruit histone methyltransferase G9a to silence NRSF target genes in nonneuronal cells (Roopra *et al*., 2004).

A DNA-binding protein PRDI-BF1, which is important in silencing multiple genes to drive terminal stage of B cell differentiation, has also been reported to associate with G9a. It recruits histone methyltransferase G9a to promoters to repress transcription of interferon-β (IFNB1) which is required for terminal stage of B cell differentiation. IFNBI gene silencing is also mediated through G9a HKMT catalytic activity (Gyory *et al*., 2004).

1.8. Muscular dystrophy

Muscular dystrophies are a group of heterogeneous genetic disorders that lead to degeneration and wasting of skeletal muscles. DMD, caused by mutations in the dystrophin gene (Koenig *et al.*, 1987), is the most prevalent muscular dystrophy that affects 1:3500 males. DMD is characterized by progressive skeletal muscle degeneration and the subsequent replacement of muscles by fibrotic and fat tissue. Patients typically are wheelchair bound in their early teens, and die in the early twenties due to respiratory and cardiac failure. The *mdx* mice lacking dystrophin have been widely studied as a mouse model for DMD and for exploring therapeutic strategies. Muscle degeneration and necrosis in *mdx* mice occurs between 3-8 weeks of age, and subsides in limb muscles thereafter, with the exception of the diaphragm that displays progressive degenerative changes and fibrosis. Despite intensive research, the mechanisms underlying muscle degeneration in DMD are largely unclear but a failure of myogenic satellite cells to maintain muscle regeneration ultimately leads to myotube necrosis.

1.8.1. Current therapy for muscular dystrophy

Muscular dystrophy is currently a major and unsolved public health problem. To date, although many kinds of studies including gene transfer strategies have been implemented, but no effective treatment for muscular dystrophy has been established. There is no known cure for muscular dystrophy and treatment is generally aimed at controlling the onset of symptoms to maximize the quality of life. Presently, only physical therapy is able to help patients maintain their muscle strength and mobile function. Besides cell-based therapies, alternative would be the use of orthopedic appliances, such as braces and wheelchairs, which improve patients' mobility and self-care ability. In some cases, patients receive surgery on the spine or legs to improve their muscle function.

The studies on various strategies for treatment of muscular diseases are still in progress. Currently, these strategies are categorized into two approaches -- Exogenous delivery and endogenous activation :

- The first approach relies on exogenous tools (gene, cell therapy) to improve muscle regeneration. Due to the availability and remarkable capacity of satellite cells to regenerate in damaged muscle, satellite cells and their descendent myoblasts have been used for stem cell-based therapies to treat muscular dystrophy. In stem cell-based therapy, myoblast transfer therapy has been proposed as a treatment for DMD, in which muscle precursor cells are transplanted directly into muscle fibers to replace damaged muscle tissues. However, donor myoblasts are less capable of cell migration and fuse poorly with recipient myotubes. Thus, the existing satellite cell-based therapy is still not an effective cure.

- The second approach consists of activating endogenous cells to stimulate myogenic factors and achieve muscle hypertrophy. Small molecule inhibitors have been identified and they play an important role in stem cell biology and regenerative medicine.

For example, some significant studies have shown that histone deacetylases (HDACs) inhibit the activities of MRFs such as MyoD and MEF2, leading to impairment of myogenic differentiation in muscle. Currently, several HDAC inhibitors have been developed and tested in clinical trials testing on the therapeutical potential in treatment of muscle and heart disease (Nebbioso *et al*., 2009). Rescue work has been carried out to prove that calcium/calmodulin-dependent protein kinase (CaMK) and calcineurin signalling can overcome the HDAC-mediated deacetylation and repression on MEF2 transcriptional activity. Treatment with a HDAC inhibitor such as Trichostatin A (TSA) induces follistatin expression which promotes hypernucleated myotubes and enhances expression of regeneration markers following muscle injury (Lezzi *et al*., 2004). In molecular study, follistatin has been shown to be able to induce satellite cells to exit from quiescence during myogenesis (Le Grand and Rudnicki 2007). Similarly, spinal muscular atrophy (SMA) mouse treated with other HDAC inhibitors such as valproic acid and phenylbutyrate also displayed enhanced differentiation of satellite cells. These treated mice exhibited better motor function, larger motor-evoked potentials, less degeneration of spinal motor neurons, less muscle atrophy, and better neuromuscular junction innervation than non-treated SMA mice (Mercuri *et al*., 2004, Brahe *et al*., 2005, Tsai *et al*., 2008a). In fact, these small molecules such as HDAC inhibitors and DNA methyltransferase inhibitors have already been proven useful in the treatment of cancer and may serve as potential pharmacological compounds for therapeutic treatment of muscular dystrophy.

However, the use of HDAC inhibitor as a therapeutic treatment for muscular dystrophy is closely monitored with caution. The effectiveness, toxicity and consequences of these drugs in human cells are not yet fully known and have to be assessed carefully.

Ironically, many approaches towards treatment for muscle disorders are not possible. Currently, understanding of the nature and fundamental cause of the disease is poor. In addition, the study of cellular and molecular events in muscle growth and regeneration is insufficient. The molecular mechanisms underlying the activation and function of the myogenic stem cells remains unclear and there is insufficient knowledge on the mechanisms underlying the inhibitors–mediated repression of MRF gene transcription in muscular disease.

Regenerative medicine requires a thorough understanding of the myogenic regulatory network controlling skeletal muscle proliferation, self-renewal and myogenic differentiation. Hence, there is a need for continuous research studies on gene regulation in muscle development and to identify repressor proteins involved in transcriptional deregulation of structural and regulatory proteins required for skeletal muscle differentiation. This will facilitate us to have a better understanding of the myogenic gene regulation at the molecular level which will allow us to develop various approaches for effective medical treatment of muscular disorders.

1.9. Perspectives and aims of study

Based on research studies, the bHLH transcription factor Sharp-1 has been identified as an inhibitor of skeletal myogenesis. Sharp-1 is over-expressed in inclusion body myositis that exhibits a differentiation defect, and is also associated with loss of skeletal muscle mass (Morosetti *et al*., 2006, Lecomte *et al*., 2010). While Sharp-1 is

overexpressed in myopathies, the mechanisms by which it regulates myogenesis are not completely understood. Besides inhibiting myogenesis, Sharp-1 also blocks adipogenic differentiation through the recruitment of a transcriptional co-factor methyltransferase G9a to adipogenic promoters (Gulbagci *et al*., 2009). The role of G9a in myogenesis is unknown and the mechanisms underlying Sharp-1-mediated repression of myogenesis are unclear. Therefore, this research aims to examine whether G9a is involved in myogenic differentiation, and once established, to investigate its relationship to Sharp-1 in the regulation of muscle cell differentiation.

The first part of the study aims to determine if G9a has a role in myogenic differentiation.

- To determine if G9a is involved in myogenic differentiation, both the expression level and cellular localisation of G9a are assessed in myoblast differentiation.
- Both gain of function and loss of function studies are used to find out if G9a has an effect on myogenic differentiation.
- In myogenic differentiation, transcription factor MyoD-mediated upregulation of p21 which correlates with cell cycle withdrawal is necessary to induce terminal skeletal muscle differentiation (Guo *et al*., 1995, Halevy *et al*., 1995). On the contrary, it was shown that G9a co-operates with CDP/cut and Gfi1 transcriptional regulators to suppress p21 expression (Nishio and Walsh, 2004, Duan *et al*., 2005). Hence, it is necessary to determine if G9a participates in the regulation of a key cell cycle regulator p21 in myogenesis. Cell proliferation status and cell cycle markers are analysed in G9a-expressing myoblasts.

- MyoD is a critical MRF that switches on differentiation genes during myogenic differentiation. One of its immediate downstream targets is myogenin. Since H3K9me (H3K9 di-methylation), a hallmark of G9a activity, associates with transcriptional repression, H3K9me levels on the myogenin promoter is assessed in G9a-expressing cells.
- The G9a SET domain confers its methyltransferase activity. To determine if the SET domain is responsible for any G9a-mediated effect in myogenesis, a comparison is made between full length G9a and G9aΔSET mutant overexpressed in myoblasts.
- In addition to deletion of the SET domain, a methyltransferase inhibitor, BIX-01294 is applied in both differentiation assay and ChIP assay to ensure that G9a methyltransferase activity is involved in G9a-mediated effect. Phenotypic effect, myogenic markers as well as H3K9me levels on the myogenin promoter are examined.
- Many studies showed that G9a is capable of methylating proteins such as such as CDYL1, WIZ, C/EBP, Reptin, ACINUS, CENP-A, CENP-B, CENP-C, TRF1 TRF2 and p53 and itself (Ueda *et al*., 2006, Chin *et al*., 2007, Rathert *et al*., 2008, Huang *et al*., 2010, Sampath *et al*., 2007, Pless *et al*., 2008, Lee *et al*., 2010). In addition to these proteins, MyoD also contains a similar methylation consensus at Lysine 104. Hence, if G9a has an effect on myoblasts differentiation, then determine if its effect is reversible and if G9a methylation of MyoD at lysine 104 is required for G9a-mediated effects in myogenesis. A rescue assay is performed with re-expression of MyoD and MyoD(K104R) in G9a-expressing cells where MyoD(K104R) is refractory to G9a methylation.

Sharp-1 inhibits myogenic and adipogenic differentiation. However, mechanisms by which Sharp-1 represses myogenesis are not well defined. Since G9a is associated with Sharp-1 in repression of adipoctyes (Gulbagci *et al*., 2009), it is interesting to find out if G9a is also involved in Sharp-1-mediated inhibition of myogenic differentiation. Hence, the second part of the study aims to determine if determine if G9a acts as a co-repressor for Sharp-1 in blocking skeletal myogenesis.

- First, to ensure that data is consistent with previous reports (Azmi *et al*., 2004), the downregulation of Sharp-1 expression is determined in myoblasts.
- For the same reason as above, Sharp-1-mediated differentiation defects is determined using differentiation assay with myoblasts overexpressing Sharp-1.
- To find out if G9a is involved in Sharp-1-mediated repression, the interaction between G9a and Sharp-1 is examined. If they interact, their interacting domains will be determined using deletion mutants. In addition, their cellular localisations will be investigated to ensure that they exist in the same compartment in a cell.
- G9a di-methylates H3K9. To find out if G9a methylation activity is involved in Sharp-1-mediated repression and whether the differentiation defects induced by Sharp-1 are similar to G9a, H3K9me levels on myogenin promoter are examined in Sharp-1 expressing cells.
- If G9a can methylate MyoD at lysine 104 as indicated in the first part of the study, then to find out if G9a methylation activity is involved in Sharp-1-mediated repression, methylation levels of MyoD and MyoD(K104R) are investigated in Sharp-1 expressing cells.
- Sharp-1 inhibits MyoD transcriptional activity. To determine if G9a activity has any relationship to Sharp-1-mediated repression, MyoD transcriptional

activity is examined in cells co-expressed with both Sharp-1 and G9a. In addition, to determine if their Sharp-1 and G9a interaction is necessary in Sharp-1 repressive effects, MyoD transcriptional activity is also examined in cells co-expressed with deleted mutants.

- It is crucial to investigate if Sharp-1-dependent repression of muscle differentiation program relies on G9a activity and expression. Experiments are carried out to determine if inhibiting G9a activity or expression in Sharp-1-expressing cells can remove repressive methylation marks on both H3K9 and MyoD and thus restore differentiation. A methyltransferase inhibitor BIX-01294 and RNAi-mediated reduction of G9a are used in Sharp-1-expressing cells.
- Re-expression of wild type MyoD rescues Sharp-1 defective myogenic differentiation (Azmi *et al*., 2004). To find out if methylation of MyoD at lysine 104 is crucial in Sharp-1 inhibitory mechanism, a comparison is made in rescue assay with re-expression of MyoD and MyoD(K104R) in Sharp-1-expressing cells, where MyoD(K104R) is insensitive to G9a methylation.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Cell culture and differentiation assays

HEK293T, NIH3T3, COS7, C3H10T½ cells and Phoenix cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic-antimycotic. C2C12 cells were cultured at low density (50% - 60% confluent) in growth medium (GM) containing DMEM supplemented with 20% FBS and 1x antibiotic-antimycotic. For differentiation assays, C2C12 cells were cultured at high density (80% - 90% confluent) in GM for a day and then switched to differentiation medium (DM, containing DMEM with 2% horse serum and 1x antibiotic-antimycotic) for another one to three days. Primary myoblasts derived from mice were plated on collagen coated plate and cultured in F-10 medium supplemented with 20% FBS and 5 ng/ml basic fibroblastic growth factor (bFGF). The following day, the medium was replaced with DMEM supplemented with 2% horse serum to induce differentiation. Human myoblast cells (Lonza) were cultured in skeletal muscle myoblast basal medium supplemented with 20% FBS (SkGM-2 BulletKit, Lonza). To induce differentiation, medium was replaced with skeletal muscle myoblast basal medium supplemented with 2% horse serum, on the following day. All cells were incubated in a 37°C humidified incubator with 5% CO2.

2.2. DNA constructs

Plasmids FLAG-G9a (1001 aa), FLAG-G9aΔANK (814 aa), FLAG-G9aΔANKSET (1-476 aa), EGFP-G9a, EGFP-G9aΔSET (1-830 aa), pBabe-G9a, MyoD and MyoD(K104R) were kindly provided by Dr. Martin J. Walsh (Mt Sinai School of Medicine, New York, USA) and Dr. Vittorio Sartorelli (National Institute of Arthritis, Bethesda, USA). GST-Sharp-1 fusion construct was previously created (Azmi *et al*.,

2003). Plasmids pBabe-Sharp-1 (cloned into pBabe-puro vector), Myc-Sharp-1 and Myc-Sharp-1 bHLH (1-112 aa) (cloned into pCS2 vector) were previously generated (Gulbagci *et al*., 2009). Myc-Sharp-1ΔO (368 aa, deletion of orange domain from 130 aa to 172 aa) and Myc-Sharp-1ΔN (1-265 aa) were generated by PCR amplification (primers and conditions listed in Table IV) and cloned into Myc-Sharp-1 in pCS2 vector with BamHI/ApaI sites and Bam HI/EcoRI sites respectively. For luciferase reporter assays, a firefly luciferase reporter construct (6E-TATA-Luc) driven by herpes simplex thymidine kinase promoter and E-Box elements (CACGTG) (Rossner *et al*., 2008), and a myogenin promoter-driven firefly luciferase reporter plasmid (pMyogLuc) (Friday *et al*., 2000) were used.

2.3. Retrovirus production and infection

Gene delivery by retroviruses is an efficient tool to overexpress a gene of interest and determine its role in a cell model. A retrovirus producer line, Phoenix cell is used for the production of retroviruses for transduction into the target cell. For protein expression using Phoenix cell, our gene of interest is cloned into the pBabe-puro retroviral vector which contains a packaging signal and an antibiotic resistance marker for selection. Phoenix cells were plated at a density of 1.5 x 10^6 cells per 100 mm-diameter dish. On the 2nd day, cells were transfected with pBabe-puro control vector (retroviral vector), pBabe-G9a or pBabe-Sharp-1 (retroviral vector containing the genes of interest) using Calcium Phosphate transfection kit (Invitrogen) according to the manufacturer's instructions. On the 3rd day, the medium was replaced with C2C12 growth medium (DMEM supplemented with 20% FBS and 1x antibiotic-antimycotic). The 1st and 2nd (the 4th and 5th day, respectively) viral supernatant containing the genes of interest, were collected and filtered through 0.45 µm filters for

transduction of target cells. The target cells, C2C12 were plated at a density of 0.3 x 10^6 cells per 100 mm-diameter dish. Next day, target cells were transduced with the respective viral supernatant in the presence of 4.8 µg/ml of polybrene. After selection with 2 µg/ml of puromycin for two days, cells are tested for overexpression of the gene of interest and its biological effects.

2.4. Transient transfection and siRNA-mediated knockdown of G9a

Cells were plated at a density of 6 x 10^5 cells per 35 mm-diameter plate and transfected the next day using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. Co-transfections of pBabe and FLAG-G9a or Myc-Sharp were carried out in a ratio of 1:9. For siRNA transfection, Sharp-1-expressing cells plated at a density of 1 x 10^5 cells per 35 mm-diameter plate were transfected with 100 nM of control scrambled siRNA (non-targeting siRNA control pool, Dharmacon) or G9a-directed siRNA (siG9a, on-target SMART pool siRNA targeting G9a**,** Dharmacon) using Lipofectamine RNAiMAX (Invitrogen). Sequences are shown in Table III. After 24 hours, cells were either harvested for immunoprecipitation assay or selected with 2 µg/ml of puromycin (Sun *et al*., 2007) for two days before plating for differentiation assay.

2.5. The methyltransferase inhibitor BIX-01294

BIX-01294 (Alexis) has been identified to inhibit G9a methyltransferase activity (Kubicek *et al*., 2007, Gazzar *et al*., 2008, Chang *et al*., 2009). In culture, cells in GM were incubated with 2.5 µM BIX-01294 for 8 hours and cells in DM were incubated with 2.5 µM BIX-01294 for another one to three days before harvesting. As controls, cells were treated with the same volume of dimethyl sulfoxide (BIX-01294 is

dissolved in 99.9% of dimethyl sulfoxide, according to the manufacturer's instruction) for the same number of days. Following, western blots, IP or ChIP analysis were carried out.

2.6. Immunofluorescence (IF) analysis

Cells were seeded onto coverslips. At the indicated time point of harvest, coverslips were rinsed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 minutes at room temperature. After three washes with PBS, the cells were permeabilized with 0.5% Triton-X-100 for 5 minutes. Cells were rinsed three times with PBS and incubated with blocking buffer (3% bovine serum albumin) for 1 hour. Cells were incubated with primary antibodies: myosin heavy chain (clone my32, Sigma, 1:200 dilution) or anti-G9a/EHMT2 (Cell signaling, 1:100 dilution), anti-DEC2 (Sigma, 1:50 dilution), anti-FLAG, anti-c-Myc (Sigma, 1:100 dilution) or anti-H3K9me2 (Millipore 1:250 dilution) for 1 hour. Cells were rinsed three times with PBS and incubated with Alexa Fluor 488 conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody (Molecular Probes, 1:250 dilution) or with Alexa Fluor 568 conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody (Molecular Probes, 1:250 dilution) for 1 hour. Cells were rinsed three times with PBS and mounted onto a slide with 2 µl DAPI (Vector laboratories). The edges of the coverslips were sealed with fingernail polish. The fluorescence images were captured using fluorescent microscope (Nikon Eclipse TE 2000-U) with 10x or 20x objective lens and MetaMorph software version 7.0r3.

2.7. Myogenic index

Myogenic index was determined by calculating the ratio of nuclei in myosin heavy chain-stained myotubes over total nuclei. At least 600 nuclei were counted from three different fields. Values were reported as means with standard deviation (shown as error bar) and represented as percentage. Statistical significance was determined using Student's *t* test and p-values were indicated with different degree of significance as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) where $p < 0.05$ were considered to be statistically significant.

2.8. Bromodeoxyuridine (BrdU)-based cell proliferation assays

C2C12 cells were plated on a coverslip at a density of 1×10^4 cells per 35 mm-diameter plate and cultured in GM or DM to induce differentiation. BrdU assay was performed as described in the manufacturer's instructions (Roche). In brief, cells were pulsed with 10 µM of BrdU for 2 hours in GM/DM. Cells were rinsed with PBS and then fixed with fixative (70% ethanol, 25% of glycine in water, pH 2) for 20 minutes at -20°C. The coverslips were rinsed with PBS for three times and then incubated with mouse anti-BrdU solution (1:100 dilution) for 45 minutes at room temperature. Following, the coverslips were rinsed with PBS for three times and then incubated with anti-mouse Ig-fluorescein (1:125 dilution) for 45 minutes at room temperature. Coverslips were rinsed with PBS for three times and mounted onto a slide with 2 µl of DAPI (Vector laboratories). The edges of the coverslips were sealed with fingernail polish. The fluorescence staining was examined under a fluorescent microscope (Nikon Eclipse TE 2000-U) with 10x objective lens and images were captured using MetaMorph software version 7.0r3. BrdU-positive-stained cells were determined and presented in percentage as means with standard deviation (shown as error bar). At

least 600 cells were calculated in three different fields. P values were determined using Student's *t* test and indicated as *($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$), indicating the level of significance.

2.9. Luciferase reporter assays

To study gene activity in skeletal muscle, luciferase reporter assays were performed using C2C12 myoblasts directly or C3H10T½ fibroblasts. C3H10T½ are fibroblasts that can be converted into myoblasts upon transfection with MyoD (Salvatori *et al*., 1995). Cells are transfected with 6E-TATA-Luc reporter or pMyogLuc reporter, Myc-Sharp-1, FLAG-G9a, MyoD and MyoD(K104R). In each transfection, the total amount (200 ng) of DNA was kept constant by addition of pCS2-empty vector. After 48 hours, cells were lysed and assayed for luciferase activity using Dual-luciferase reporter assay system (Promega). Luciferase activity was measured using Tecan microplate reader and Magellan 6 software. Each transfection was performed in triplicates. Values were reported as means with standard deviation (shown as error bar). Calculated p values using Student's *t* test were indicated as * with different level of significance, * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$) where $p < 0.05$ was considered to be statistically significant.

2.10. Western blot analysis and antibodies

At the indicated time point of harvest, cells were lysed with RIPA lysis buffer (1% Triton X-100, 1 mM EDTA, 50 mM Tris-HCL pH8, 50 mM NaCl, 0.1% sodium deoxycholate and 1x Protease inhibitor). Cells were scraped off from culture plates containing lysis buffer and transferred into centrifuge tubes. After 30 minutes, cell debris was removed by centrifugation at 13,000 rpm for 10 minutes at 4°C. The

protein concentrations were determined using Bradford protein assay reagent (Bio-Rad laboratories) and spectrophotometer (Shimadzu BioSpec-1601). Proteins were denatured in sample buffer by boiling for 5 minutes at 95°C. Proteins were separated on (8% - 12%) of SDS-PAGE gels and electrophoretically transferred onto a nitrocellulose membrane for western blot assay. The membrane was blocked with 5% milk in PBS Tween and then incubated with primary antibodies : anti-MyoD (M-318, Santa Cruz Biotechnology, dilution 1:200), anti-myogenin (M225, Santa Cruz Biotechnology, dilution 1:200), anti-troponin T (Sigma, dilution 1:1000), anti-cyclinD1 (H295, Santa Cruz Biotechnology, dilution1:100), anti-p21 (C-19, Santa Cruz Biotechnology, dilution 1:400), anti-FLAG (Sigma, dilution 1:1000), anti-c-Myc (Sigma, dilution 1:1000), anti-G9a/EHMT2 (Cell signalling, dilution 1:1000) or anti-β-actin (Sigma, dilution 1:10000). Following, the membrane was incubated with either horseradish-peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies. Western blots were developed using ECL Plus western blotting detection reagents (Amersham Biosciences).

2.11. Immunoprecipitation (IP) Assays

500 µg of cell lysates were incubated with 20 µl anti-c-Myc agarose beads or anti-FLAG beads (Sigma) rotating overnight at 4^{o}C. Alternatively, 500 µg -1000 µg of cell lysates were first incubated with 2 µg of antibody rotating overnight at 4^{o}C and then incubated with 20 µl of A/G agarose beads (Santa Cruz) for 2 hours at 4^{o}C with rotation. Next, IP beads were washed three times with RIPA lysis buffer rotating at 4^{o}C for 5 minutes for three times. Lysis buffer was discarded and IP beads were boiled in 20 µl of 2x SDS-PAGE loading buffer. The samples were analysed by SDS-PAGE and western blot.

2.12. GST-pull down assays

GST-Sharp-1 and GST control proteins were expressed in *Escherichia coli* BL21 cells with Luria-Bertani (LB) medium incubated at 37^{o}C until the OD at 600nm reached 0.8. These proteins were induced with 0.5 mM IPTG at 37^{o}C for 3 hours. Next, cells were centrifuged at 5000 rpm for 10 minutes. Supernatant were discarded and cell pellets were resuspended in PBS buffer containing 1x PBS, 1% Triton X-100, 0.1 mM Dithiothreitol (DTT) and complete protease inhibitor cocktail (Roche). The solution was subjected to sonication in ice bath (10 seconds sonication/ 15 seconds pause/ 5 cycles). After centrifugation at 10,000 rpm for 10 minutes at 4^{o}C, the soluble fractions were incubated with glutathione sepharose 4B beads for 2 hours at 4^{o}C with rotation. Beads were centrifuged at 2500 rpm for 2 minutes at 4^{o}C. Supernatant were discarded and beads were resuspended in PBS buffer as described above to make a 50% slurry. GST proteins were quantified by SDS-PAGE and coomassie blue staining. At the same time, *in vitro* translated protein was prepared using TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. 10 µg of GST proteins or GST-Sharp-1 were incubated with 20 µl of *in vitro* translated FLAG-G9a protein in 200 µl of binding buffer (50 mM Tris-HCL pH8.0, 100 mM NaCl, 0.3 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, protease inhibitor) rotating for 2 hours at 4^{o}C. The beads were washed three times with binding buffer before resuspended in SDS-PAGE loading buffer. The samples were analysed by SDS-PAGE and western blot. Anti-FLAG (Sigma, dilution 1:1000) primary antibody and subsequently horseradish-peroxidase-conjugated anti-mouse IgG secondary antibody were used in this GST assay.

2.13. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total mRNA was isolated using TRIZOL (Invitrogen) according to the manufacturer's instructions. cDNA were made using Avian Myeloblastosis Virus (AMV) Reverse transcriptase (Promega) according to the manufacturer's instructions with 4 µg of RNA and 1 µl of oligo (dT) as the primer. cDNA were normalised with 36B4. Normalised cDNA were used to analyse G9a, MyoD and myogenin mRNA levels. Primers sequences are shown in Table I. PCR was carried out using the GoTaq Flexi DNA Polymerase (Promega), 1x buffer, 1.5 mM Magnesium Chloride, 0.1 mM deoxyribonucleotide triphosphate (dNTPs), 0.5 µg template, 0.1 µM forward primer, 0.1 µM reverse primer and water in a total volume of 25 µl. PCR reaction were loaded into a PCR thermal cycler under the following conditions : 1 cycle of 95°C for 5 minutes, followed by indicated number of cycles (as shown in Table II) of 95°C for 1 minute**,** 58°C for 45 seconds, 72°C for 1 minute, and ending with 72°C for 10 minutes. PCR products were analysed by agarose gel electrophoresis using a (1% - 3%) agarose gel in a 1x Tris-acetate-EDTA (0.04M Tris-acetate, 0.001M EDTA) buffer. Reverse-transcribed cDNA were also quantified by real-time PCR.

2.14. Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were performed as described by the manufacturer (Millipore). Briefly, cells were fixed in 1% formaldehyde for 10 minutes at 37°C, washed in PBS and scraped off from culture plate. Cells were resuspended in SDS Lysis Buffer (50 mM Tris-Cl pH 8.1, 1% SDS, 10 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A). Cells were sheared by sonication to obtain DNA fragments and the chromatin were immunoprecipitated with 2 µg antibodies directed against

H3K9me2 (histone 3 di-methyl Lysine 9, Upstate), H3K9K14ac (histone 3 acetylated lysine 9 and lysine 14, Upstate). The cross-links were heat-reversed (65°C) and DNA was purified using phenol-chloroform. DNA level was quantified by Q-PCR using primers specific to myogenin promoter sequence and β-actin as described in Table II. Each sample was performed in triplicate and the values obtained were normalised to β-actin. The results were presented as average with standard deviation (shown as error bar). P-values were calculated using Student's t-test and presented with different level of significance as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). It is important to note that ChIP assay measures the amount of a chromatin fraction containing a specific antigen relative to the total amount of initial chromatin. Hence, between repeated experiments, the ChIP-binding patterns are similar but the enrichment values differs due to varying amounts of chromatin applied or different hybridization efficiency.

2.15. Quantitative real-time polymerase chain reaction (Q-PCR)

The cDNA was amplified using Lightcycler 480 SYBR Green 1 Master Kit (Roche). Samples were loaded into Roche Light Cycler 480 (LC480) instrument and light cycler 480 software (version 1.3.0.0705) was used for analysis. Primers specific to G9a, Sharp-1 and GAPDH are shown in Table II.

Chapter 3

Results

3. Results

3.1. G9a inhibits myogenic differentiation

3.1.1. Expression of G9a decreases upon myogenic differentiation

Previously, Sharp-1 has been shown to inhibit both myogenesis (Azmi *et al*., 2004) and adipogenesis (Gulbagci *et al*., 2009). During Sharp-1-mediated repression of adipogenesis, methyltransferase G9a was recruited onto C/EBPα and PPARγ2 promoters to regulate adipocytes differentiation. To investigate if G9a is also involved in Sharp-1-mediated inhibition of myogenesis, it was necessary to first examine if G9a has any role in the regulation of myogenesis. As a start point, G9a expression was examined in proliferating and differentiating C2C12 myoblasts. C2C12 cells were cultured and harvested at day 0 (proliferative phase), day 1 and 3 (differentiated phase). Total RNA were collected and the mRNA levels of G9a, MyoD, myogenin were reverse transcribed and analysed by quantitative reverse transcription polymerase chain reaction (QRT-PCR) or semi-quantitative RT-PCR. The values obtained were normalised against GAPDH mRNA levels and are presented as relative G9a mRNA levels (Figure 3.1.1.A). The cDNA samples were also amplified by conventional PCR using primers for G9a, MyoD, myogenin and 36B4 genes (primer sequences are shown in table I). The amplified PCR products were then analysed on agarose gel electrophoresis (Figure 3.1.1.B). Expression of 36B4 was used as a loading control. In both Q-RT-PCR and conventional PCR analysis, G9a mRNA was expressed in C2C12 cells in the proliferating myoblasts (72.2%), and its expression declined rapidly during differentiation (17.1%, 15.1% and 14.9% for day 1, 2, 3 respectively). The increased expression of MyoD and myogenin served as a control differentiation. In addition, the G9a protein expression was examined by western blot in C2C12 cells. β-actin expression was used as an internal control (Figure 3.1.1.C).

Similar to mRNA expression pattern, the protein expression of G9a was reduced upon induction of differentiation (74.4%, 25.3% and 7.4% for day 0, 1 and 3, respectively), whereas the expression of myogenic proteins, MyoD, myogenin and troponin T increased.

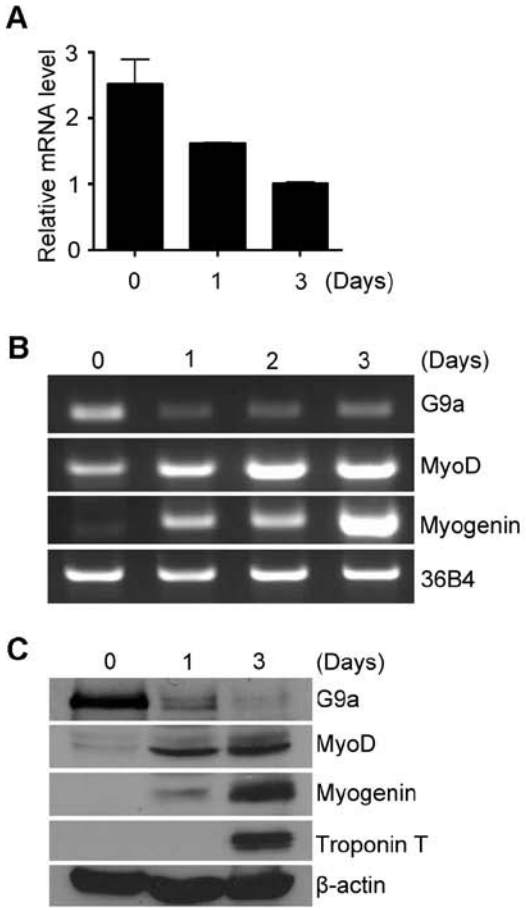

Figure 3.1.1 Expression of G9a in C2C12 myoblasts.

The G9a mRNA levels were examined in proliferating (day 0) and differentiating (day 1, 3) C2C12 cells. The relative mRNA expression was quantified and standardised against the house-keeping gene GAPDH. Values were presented as mean with standard deviation (error bar) for three independent experiments (A). The mRNA levels of G9a, myogenic markers MyoD and myogenin, and 36B4 (internal control) in proliferating (day 0) and differentiating (day 1, 2, 3) C2C12 cells were examined

using semi-quantitative RT-PCR (B). Protein expression levels of G9a, MyoD, myogenin, troponin T were also analysed by western blot. β-actin was used as an internal control (C).

3.1.2. Expression of G9a in primary mouse myoblasts decreases upon myogenic differentiation

In order to further validate the expression of G9a, both semi-quantitative and real-time RT-PCR were performed using primary mouse myoblasts (Figure 3.1.2.A, B). The expression of G9a in differentiating primary mouse myoblasts also showed the same decline as in C2C12 myoblasts.

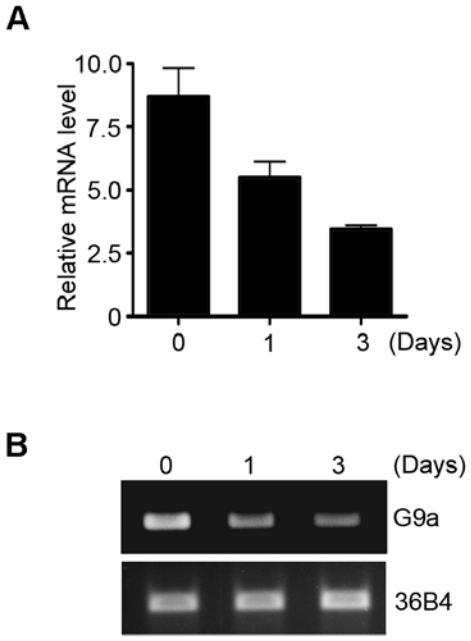

Figure 3.1.2 Expression of G9a in primary mouse myoblasts.

G9a mRNA expression levels during primary mouse myoblast differentiation were determined. The mRNA levels were normalised to GAPDH and quantified by QRT-PCR (A) as well as normalised to 36B4 and analysed by semi-quantitative RT-PCR

(B). Values for Q-PCR are presented as means with standard deviation (error bar) for three independent experiments.

3.1.3. G9a remains in the nucleus during myoblast differentiation

The expression of G9a declines as myoblasts differentiate but the localisation during differentiation was unclear. Hence, the localisation of G9a protein in myoblast cells undergoing proliferation and differentiation was examined by immunofluorescence microscopy. C2C12 cells, cultured in growth medium (GM, proliferation phase) or in differentiating medium (DM) for three days to induce differentiation, were fixed for immunofluorescence staining. Endogenous G9a was stained with FITC-conjugated antibody (green), myosin heavy chain (MHC) was stained with Texas-Red-conjugated antibody and nuclei were stained with DAPI. Fluorescence images were captured using a 20x objective lens (Figure 3.1.3). G9a was expressed in the nucleus in proliferating C2C12 myoblasts and its expression remained nuclear in differentiated cells expressing myosin heavy chain. Similar to earlier results, G9a expression was lower in differentiated cells compared to proliferating cells. All the results showed that G9a declined during differentiation of myoblasts, suggesting that it may be crucial to maintain myoblasts in a proliferative phase.

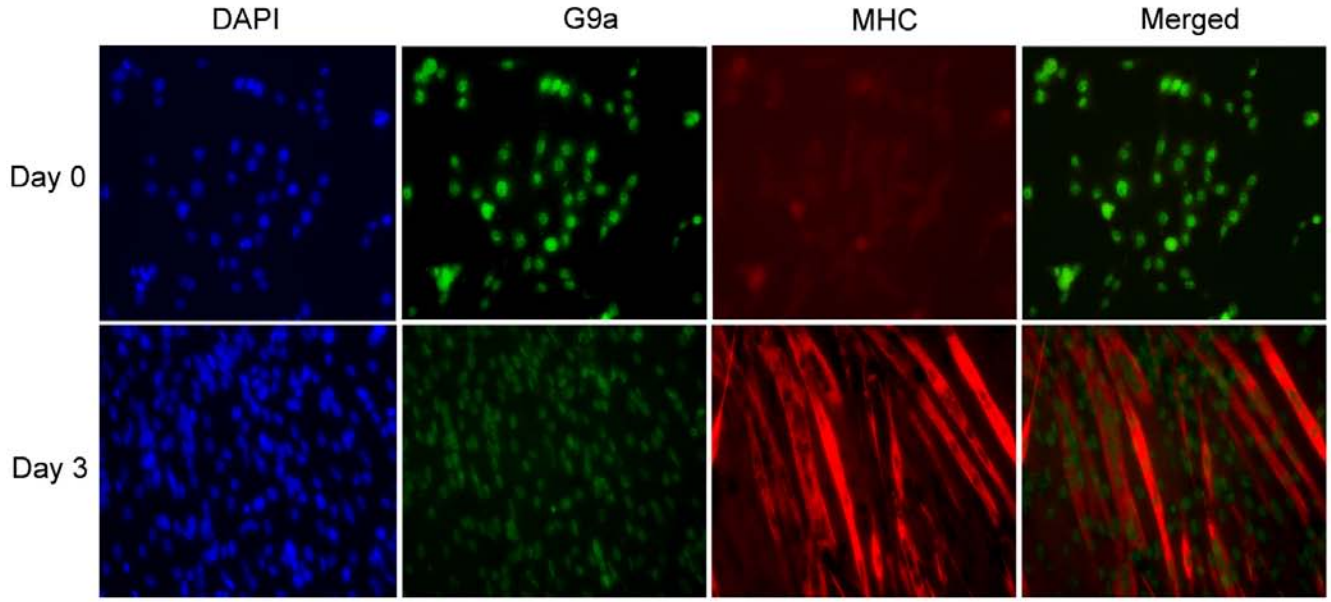

Figure 3.1.3 Localisation of G9a in undifferentiated and differentiating myoblasts.

Localisation of endogenous G9a in day 0 (cells in growth phase) and in day 3 (cells in differentiation phase) were observed by immunofluorescence microscopy. Differentiated cells were identified by immunofluorescence staining with anti-myosin heavy chain antibody (MHC, red). Nuclei were immunostained with DAPI (blue) and cellular localisation of G9a (green) was observed. The fluorescence images were captured under a microscope using 20x objective lens and MetaMorph software.

3.1.4. Overexpression of G9a inhibits myoblast differentiation

G9a belongs to the methyltransferase family which includes Suv39h1, ESET/SETDB1 and Ezh2. Suv39h1 and Ezh2 are down-regulated during myoblast differentiation and their overexpression have been reported to inhibit skeletal muscle differentiation (Caretti *et al*., 2004; Mal *et al*., 2006). As G9a expression also declined during myogenic differentiation, I examined if G9a plays a role in myogenesis. A gain of function study was done to observe the impact of G9a in differentiation. Phoenix retroviral producer cells were used to generate pBabe- (puromycin resistant control

vector) or pBabe-G9-expressing retroviruses which were then used to transduce C2C12 myoblasts. Overexpression of G9a was determined by western blot analysis (Figure 3.1.4.A). C2C12 cells were left untransfected or retrovirally transduced either with pBabe or pBabe-G9a. Untransfected or puromycin-selected transduced cells were cultured in growth medium (GM) or in differentiating medium (DM) for one day or three days. Morphological changes of cells was observed during their proliferation phase (undifferentiated cells at day 0) and differentiation phase (day 1 and day 3) under a phase contrast microscope using 10x (Figure 3.1.4.B) and 20x objective lens (Figure 3.1.4.C). Morphologically, cell alignment was observed on day 1 in untransfected (control) and pBabe-expressing cells (control) but not in pBabe-G9a-expressing cells (Figure 3.1.4.D). Moreover, the number of myotubes was found to be distinctly reduced in pBabe-G9a-expressing cells compared with untransfected or pBabe-expressing cells. These results suggest that G9a has a role in myogenic differentiation and its overexpression inhibits myoblast differentiation and myotube formation.

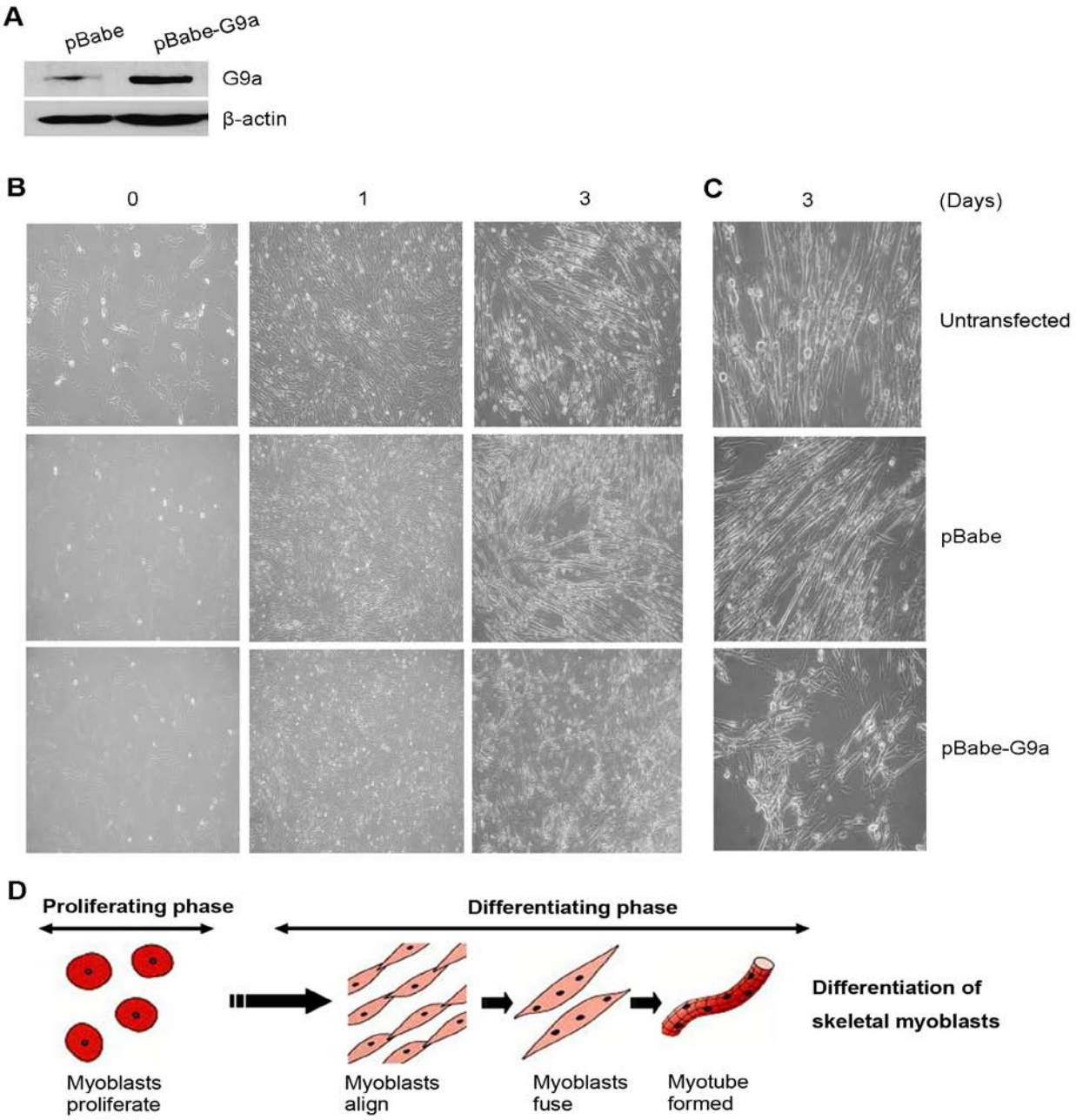

Figure 3.1.4 Overexpression of G9a inhibits myogenic differentiation.

Phoenix cells were transfected with pBabe (vector control) or pBabe-G9a and analysed for the expression of G9a. β-actin was used as a loading control (A). C2C12 cells were left untranfected or transduced with either pBabe- or pBabe-G9a-encoding retrovirus. Untransfected or transduced cells were cultured in GM followed by one to three days in DM to induce to differentiation. Morphological analysis of myogenic differentiation were analysed and captured at 10x magnification (B) or 20x magnification (C). A schematic representation of skeletal myoblast differentiation,

showing myoblast proliferation, myoblast alignment, myoblast fusion and myotube formation (D).

3.1.5. Overexpression of G9a inhibits expression of muscle-specific genes

To further verify that G9a inhibits myogenic differentiation, the formation of multinucleated myotubes was determined by immunostaining with MHC, a marker of differentiated myotubes. C2C12 cells-expressing pBabe and pBabe-G9a were cultured to proliferate in GM (day 0) or differentiate in DM for one to three days (day 1, 3). At the respective time points, undifferentiated and differentiated cells were fixed and stained with monoclonal MHC antibody, followed by anti-mouse Texas-red conjugated secondary antibody. The nuclei were stained with DAPI. MHC expression and myotube formation was severely impaired in C2C12 cells overexpressing G9a in day 1 and 3 after differentiation (Figure 3.1.5.A), demonstrating that G9a inhibits MHC expression and myogenic differentiation.

To further substantiate these findings at the molecular level, expression of myogenic markers was assessed in pBabe- and pBabe-G9a-expressing cells. pBabe- and pBabe-G9a-expressing C2C12 cells were cultured in GM or in DM. Undifferentiated cell lysate (day 0) and differentiated cell lysates (day 1, 3) were collected and subjected to western blot analysis. Protein levels of myogenic differentiation markers myogenin, troponin T were examined and β-actin was used as a loading control. The expression of myogenin expression was reduced in pBabe-G9a (1.94% and 24.55% at day 1 and 3 respectively), compared with pBabe controls (7.65% and 27.6% at day 1 and 3 respectively). Similarly, troponin T levels was obviously reduced in differentiating cells expressing G9a (Figure 3.1.5.B). These biochemical changes are consistent with morphological and immunofluorescence with MHC staining. Thus, G9a inhibits

expression of myogenic differentiation markers and blocks skeletal muscle differentiation.

To examine whether G9a has an impact in primary myoblasts, similar experiments were carried out using primary cultures of normal human skeletal myoblasts Human primary myoblasts were infected with pBabe- or pBabe-G9a encoding retrovirus. The human myoblasts overexpressing pBabe or pBabe-G9a were cultured to proliferate in growth medium or differentiate in differentiation medium for three days. Cell lysates were collected and subjected to western blot analysis using antibody against G9a, troponin T and β-actin (serve as a loading control). Expression of G9a was determined (Figure 3.1.4.A) and expression of skeletal muscle differentiation marker troponin T was examined (Figure 3.1.5.C). In human myoblasts overexpressing G9a, the terminal differentiation marker, troponin T was reduced. This result further validates the earlier tests in transformed C2C12 cells and confirms that G9a suppresses skeletal muscle differentiation.

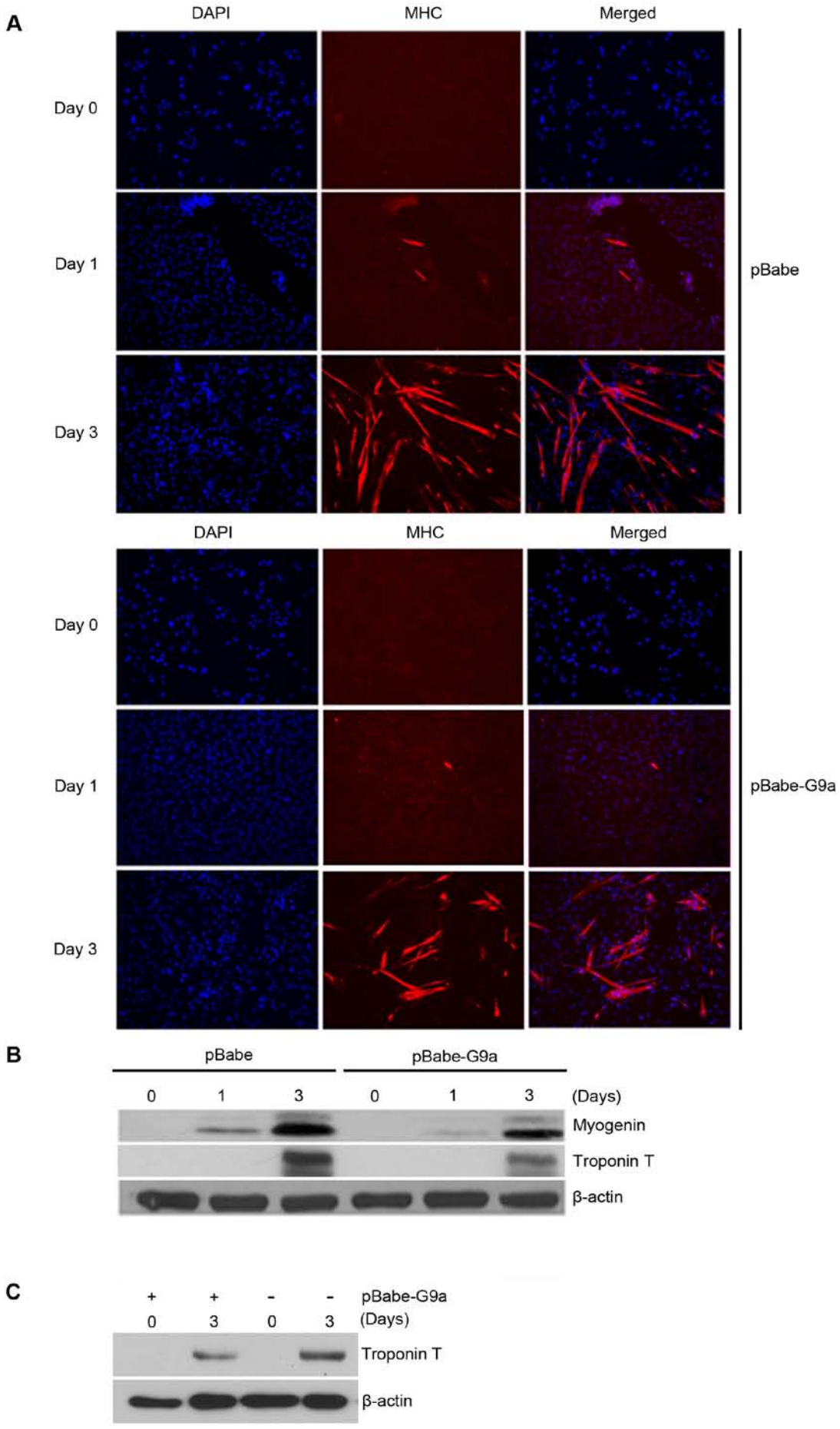
A
DAPI
MHC
Merged
Day 0
Day 1
Day 3
pBabe
DAPI
MHC
Merged
Day 0
Day 1
Day 3
pBabe-G9a
B
pBabe
pBabe-G9a
0
1
3
0
1
3
(Days)
Myogenin
Troponin T
β-actin
C
+
+
-
-
pBabe-G9a
0
3
0
3
(Days)
Troponin T
β-actin

Figure 3.1.5 G9a inhibits differentiation and expression of muscle-specific genes. pBabe (vector control) and pBabe-G9a infected C2C12 cells were cultured to proliferate (day 0) or induced to differentiate (day 1, 3). The extent of differentiation was assessed by immunofluorescence staining with myosin heavy chain (MHC, red). The nuclei were stained counterstained with DAPI (blue). The images were merged and captured at 10x magnification (A). Cell lysates were analysed by western blot for myogenic markers myogenin and troponin T. β-actin was used as loading control (B). pBabe- (vector control) and pBabe-G9a-expressing human primary myoblasts were cultured in GM or induced to differentiate for three days. Cell lysates were analysed by western blot for troponin T expression (C). β-actin was used as loading control.

3.2. G9a inhibits cell-cycle progression

3.2.1. Overexpression of G9a promotes myoblast proliferation

During myoblast differentiation, MyoD upregulates the expression of the cyclin-dependent kinase (Cdk) inhibitor p21. MyoD-mediated induction of p21 correlates with cell cycle withdrawal and cell cycle arrest which is necessary to induce terminal skeletal muscle differentiation (Guo *et al*., 1995, Halevy *et al*., 1995). However, reports have shown that methyltransferase G9a can also suppress the cell cycle inhibitor p21 which leads to a delayed cell cycle exit (Nishio and Walsh, 2004, Duan *et al*., 2005). Together, these suggest the likelihood that G9a may also suppress p21 which will delay cell cycle exit and hence prevent skeletal muscle differentiation. Since overexpression of G9a inhibited skeletal muscle differentiation, it was not clear if this was due to an impact of G9a in delaying cell cycle exit and thus impairing cell differentiation. To elucidate if G9a affects cell proliferation, bromodeoxyuridine (BrdU) incorporation assay was performed. Cells that are in S-phase of the cell cycle will incorporate BrdU and an analysis of the BrdU-positive signals provides an estimate for the fraction of cells in S-phase. C2C12 cells overexpressing pCS2 (vector

control) or FLAG-G9a were cultured in GM and pulse-labelled with BrdU. BrdU pulse-labelled cells were fixed and immunostained with mouse anti-BrdU antibody, followed by anti-mouse Ig-fluorescein. Fluorescence images showed more BrdU-positive cells in cells overexpressing G9a as compared to control cells (Figure 3.2.1.A). The percentage of BrdU-positive cells was calculated by counting at least 600 cells and represented in graph as mean with standard deviation (Figure 3.2.1.B). The statistical results showed that significantly, overexpression of G9a induced a higher rate of cell proliferation, indicating that G9a promotes myoblast proliferation.

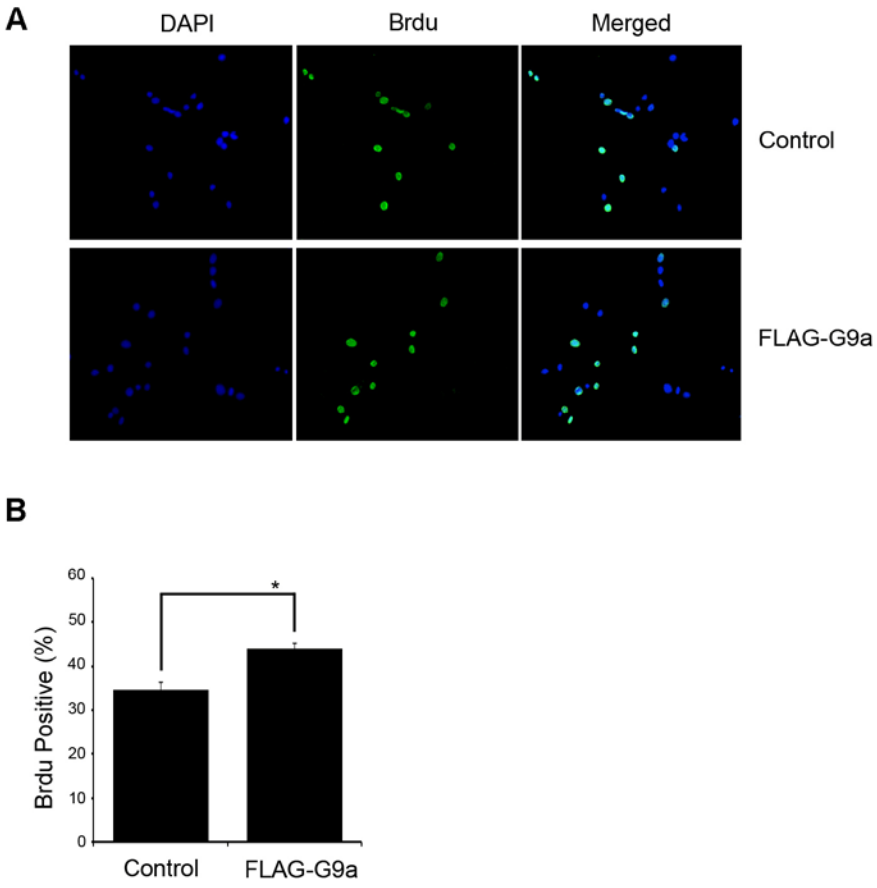

Figure 3.2.1 Overexpression of G9a promotes myoblast proliferation.

C2C12 cells were transfected with pCS2 (vector control) or FLAG-G9a and cultured in growth medium. Cells were pulsed-labeled with BrdU and immunostained with

anti-BrdU antibody. BrdU-positive cells (green) and DAPI-stained nuclei (blue) were examined under a fluorescent microscope with a 20x objective lens (A). The percentage of BrdU-positive cells was calculated and presented as means with standard deviation (error bar). P-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant). This experiment was carried out at least twice with similar results (B).

3.2.2. G9a delays cell cycle exit during differentiation

During differentiation, myoblasts are required to withdraw from the cell cycle in order to express terminal differentiation markers. However, not all the cells terminally differentiate but a small population of myoblasts remain undifferentiated. Hence, myoblasts are thought to exist both in a quiescent and differentiated state with a low proliferative index (Baroffio *et al*., 1996, Yoshida *et al*., 1998). To further investigate if G9a continues to promote cell cycle progression, similar BrdU incorporation assay was performed to assess the proliferative capacity of cells cultured under differentiating conditions, unlike the previous cells cultured under proliferating condition. Unlike in proliferative state, C2C12 cells overexpressing pCS2 (vector control) or FLAG-G9a were cultured to about 80% confluence and then subjected to differentiation conditions. The cells were pulse-labelled with BrdU and a green fluorescent dye. Fluorescence images revealed that there were more BrdU-positive-stained cells in G9a-expressing cells than in control cells undergoing differentiation (Figure 3.2.2.A). The percentage of BrdU-positive-stained cells was determined, by counting the number of BrdU-positive stained cells over a total of at least 600 cells (Figure 3.2.2.B). There were significantly more BrdU-positive cells in G9a-expressing cells (23.8%) compared to control cells (15.4%). Thus, G9a plays a role in

promoting cell proliferation and delays cell cycle exit which probably leads to a delay in myogenic differentiation.

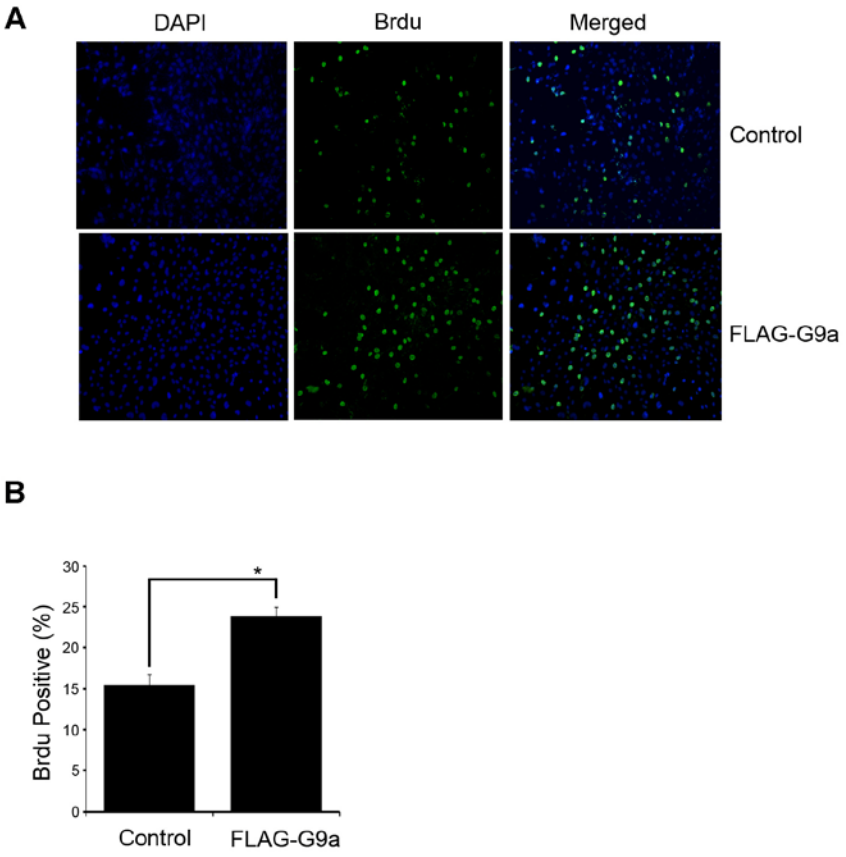

Figure 3.2.2 G9a delays cell cycle exit during myoblast differentiation.

pCS2- (control vector) or FLAG-G9a-expressing C2C12 cells were induced to differentiate for one day and then pulsed-labeled with BrdU. Cells were immunostained with anti-BrdU antibody (green) and analysed under a microscope with 20x magnification. Nuclei were stained with DAPI (blue) (A). The percentage of BrdU-positive cells were determined and presented as means with standard deviation (error bar). The p-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant). This experiment was carried out at least twice showing similar results (B).

3.2.3. G9a suppresses p21 expression

Previous studies have shown that G9a is recruited by the transcription regulator CDP/cut (CCAAT displacement protein_*cut* homolog), Gfi (growth factor independent 1) and protein UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) to block p21 expression and enhances cell cycle progression (Nishio and Walsh, 2004, Duan *et al*., 2005, Kim *et al*., 2009). To examine if the alteration in cell cycle exit in myoblast is due to G9a-mediated transcriptional repression of p21, C2C12 cells overexpressing EGFP (vector control) or EGFP-G9a were cultured in both proliferative phase and differentiation phase for one to three days. Cell lysates were collected and subjected to western blot analysis for p21 and cyclin D1 (Figure 3.2.3.). In G9a-expressing cells, the level of p21 expression was clearly reduced as cells differentiated. Conversely, the expression of cyclin D1 which is required for G1 phase cell cycle progression was relatively higher at day 1. The reduction in p21 expression and the increase in cyclin D1 expression correlate with the BrdU assay which demonstrates that G9a enhances myoblast proliferation and delays cell cycle exit in C2C12 differentiation.

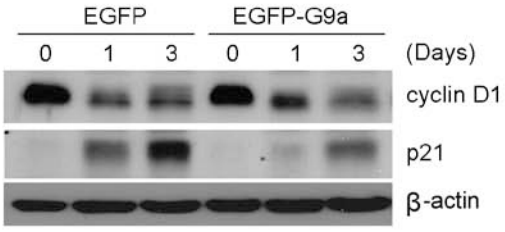

Figure 3.2.3 G9a suppresses p21 expression and cell-cycle progression.
C2C12 cells overexpressing EGFP (vector control) or EGFP-G9a were cultured to proliferate (day 0) or induced to differentiate (day 1, 3). Cell lysates were collected

and analysed by western blot for cell-cycle markers cyclin D1 and p21. β-actin was loaded as a control.

3.3. G9a represses transcription through its methyltransferase activity

3.3.1. Overexpression of G9a increases H3K9me2 mark on the myogenin promoter

G9a has been shown to preferentially di-methylate histone 3 lysine 9 (Tachibana *et al*., 2002, Rice *et al*., 2003) which correlates with transcriptional repression. To examine if there is a link between G9a methyltransferase activity and transcriptional repression of muscle-specific genes, chromatin Immunoprecipitation (ChIP) assay was performed to assess H3K9me2 changes on the myogenin promoter. EGFP (vector control) and EGFP-G9a expressing C2C12 cells were cultured to proliferate (undifferentiated day 0) and differentiate for two days (differentiated day 2). Cell lysates were subjected to sonication to shear the chromatin and then immunoprecipitated with H3K9me2 antibody. In undifferentiated control cells, myogenin promoter was H3K9me2 which declined upon differentiation. This is consistent with data shown in other reports (Mal *et al*., 2002, Caretti *et al*., 2004). G9a-expressing cells, on the other hand, had significantly higher H3K9me2 levels on myogenin promoter in both undifferentiated and differentiated cells (Figure 3.3.1.A). Thus, overexpression of G9a leads to an increase of H3K9me2 levels at the myogenin promoter which correlates with the repression in muscle differentiation gene expression and explains the differentiation defects.

A similar ChIP experiment was repeated with EGFP- and EGFP-G9a-expressing C2C12 cells using acetylated H3K9K14ac antibody to assess the level of acetylated histones, since a reduction in histone acetylation correlates with reduced gene

expression. Acetyl histone H3K9K14 levels were significantly lower in G9a expressing cells compared to control cells (Figure 3.3.1.B).

Therefore, the methyltransferase G9a increases H3K9me2 and reduces H3K9K14ac on the myogenic gene promoter which precedes the decrease in the expression of myogenic markers necessary for myotube formation in myoblast differentiation.

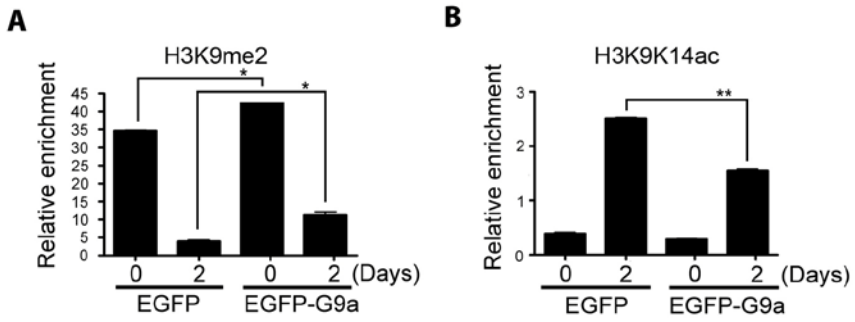

Figure 3.3.1 G9a alters histone H3K9me2 and H3K9K14ac levels on myogenin promoter.

EGFP- (vector control) or EGFP-G9a-expressing C2C12 cells were cultured to proliferate or induced to differentiate for two days. Cells were analysed by ChIP assays to determine the H3K9me2 (A) and H3K9K14ac (B) levels on the myogenin promoter. Cell lysates were subjected to immunoprecipitation with H3K9me2 or H3K9K14ac antibody and the chromatin were analysed by QPCR using primers for the myogenin promoter and beta-actin. The experiments were carried out at least twice with values represented as means with standard deviation (error bar). P-values were calculated using Student's t-test (* $p < 0.05$, significant, ** ($p < 0.01$, highly significant).

3.3.2. The catalytic SET domain in lysine methyltransferase G9a

Lysine methyltransferase has a SET domain that is responsible for methylating lysine residues on histones. The SET domain in both Suv39H1 and Ezh2 are essential for the

muscle differentiation defect (Caretti *et al*., 2004, Mal, 2006). The human methyltransferase G9a gene contains ankyrin repeats and a catalytic SET domain. The ankyrin repeats are approximately 186 amino acids and the SET domain is approximately 115 amino acids. G9a SET domain exhibits high sequence similarity to other methyltransferases, Suv29h1 andEzh2 SET (Figure 3.3.2.).

HKMT	Met Sites	Amino acid sequences
SUV39H1 SET domain	H3K9	242- RYD**L**CIF**RT**DDGR**GWGVRTL**EK**I**RKNS**F**VM**EYVGEII**TSE**EA**ER**R**--QIYDRQGAT**YLF** 300-**DLD**YVE-----D**VY**TV**DA**A**YYGN**--------**IS**H**F**V**NH**S**CDPN**LQVYN**V**FIDNL**DE**R**L**PR**IAFF**A 353-T**RT**I**RAGEELTFDY**NMQV
Ezh2 SET domain	H3K27	610- SKKHLLLAPSDVA**GWG**IFIKDPVQKNE**F**IS**EY**C**GEII**SQD**EA**DR**R**GKVYDKYMC--**FLF** 668-**NLN**NDF--------**V**V**DA**TRK**GN**--------K**IR**F**AN**H**SV**NPN**CYAK **V**MMVNG**D**H**R**----**I**GI**F**A 714-K**RAI** QT**GEELFFDY**RYSQ
G9a SET domain	H3K9	827-KVR**L**QLY**RT**AK-M**GWGVRAL**QT**I**PQGT**F**IC**EYVGEL**I**SDA**EA**DV**R**--------EDDS**YLF** 879-**DLD**NKDG----E**VY**CI**DAR YYGN**--------**IS**R**F**I**NH**L**CDPN**IIPVR**V**FMLHQ**D**L**R**FPR**IAFF**S 933-S**RD**IRT**GEEL**G**FDY**GDRF

Figure 3.3.2 SET domains in methyltransferases.

The protein sequences of the SET domain in various histone methyltransferases (HKMT) with indication of their methylation (met) sites. Identical amino sequences of the SET domain are indicated as residues in bold.

3.3.3. The SET domain in G9a is required to inhibit differentiation

A mutagenesis study was necessary to investigate if G9a SET domain is also required for the inhibition of skeletal muscle differentiation. The full length G9a encodes a protein product of 1001 amino acids whereas the G9aΔSET mutant , has its SET domain deleted, encodes a protein product of 830 amino acids (Figure 3.3.3.A). Both the wild type and deletion constructs were used in differentiation assay to determine if catalytic SET domain is involved in regulating myogenesis.

Many studies have established that the SET domain in G9a is responsible for H3K9me2 which is associated with gene silencing. In addition, the SET domain in other methyltransferases Suv39H1 and Ezh2 were reported to be necessary for the inhibition of muscle differentiation (Caretti *et al*., 2004, Mal, 2006). To examine if the SET domain in G9a is necessary for inhibiting myogenic differentiation, a differentiation assay was performed with both wild type G9a and G9aΔSET mutant. C2C12 cells were cotransfected with EGFP-G9a or EGFP-G9aΔSET mutant and pBabe vector at a ratio 9:1. After selection with puromycin for two days, transfected cells were cultured for differentiation assay and cell lysates were collected for western blot analysis. The protein expression of both EGFP-G9a and EGFP-G9aΔSET were detected in C2C12 cells (Figure 3.3.3.B). In addition, the undifferentiated cells (day 0) and differentiated cells (day 3) were immunostained with MHC antibody, followed by Texas-red conjugated antibody while the nuclei were stained with DAPI. Under the differentiation conditions, G9a-expressing cells exhibited a reduction in expression of MHC-positive cells and differentiated myotubes compared to control cells. However, G9aΔSET-expressing cells did not show differentiation defect (Figure 3.3.3.C), indicating that the SET domain in G9a appears necessary for inhibition of myotube formation and cell differentiation. To quantify the differentiation defects, myogenic index was determined. Myogenic index is defined as the ratio of the number of nuclei within the MHC-positive myotubes over a total of at least 600 nuclei. The myogenic index in EGFP-, EGFP-G9a- and EGFP-G9aΔSET-expressing cells was calculated and presented as mean with standard deviation. This statistical study showed that there was a significant ($p < 0.01$) reduction in the level of MHC expressed in G9a-expressing cells as compared to control cells and G9aΔSET-expressing cells (Figure 3.3.3.D).

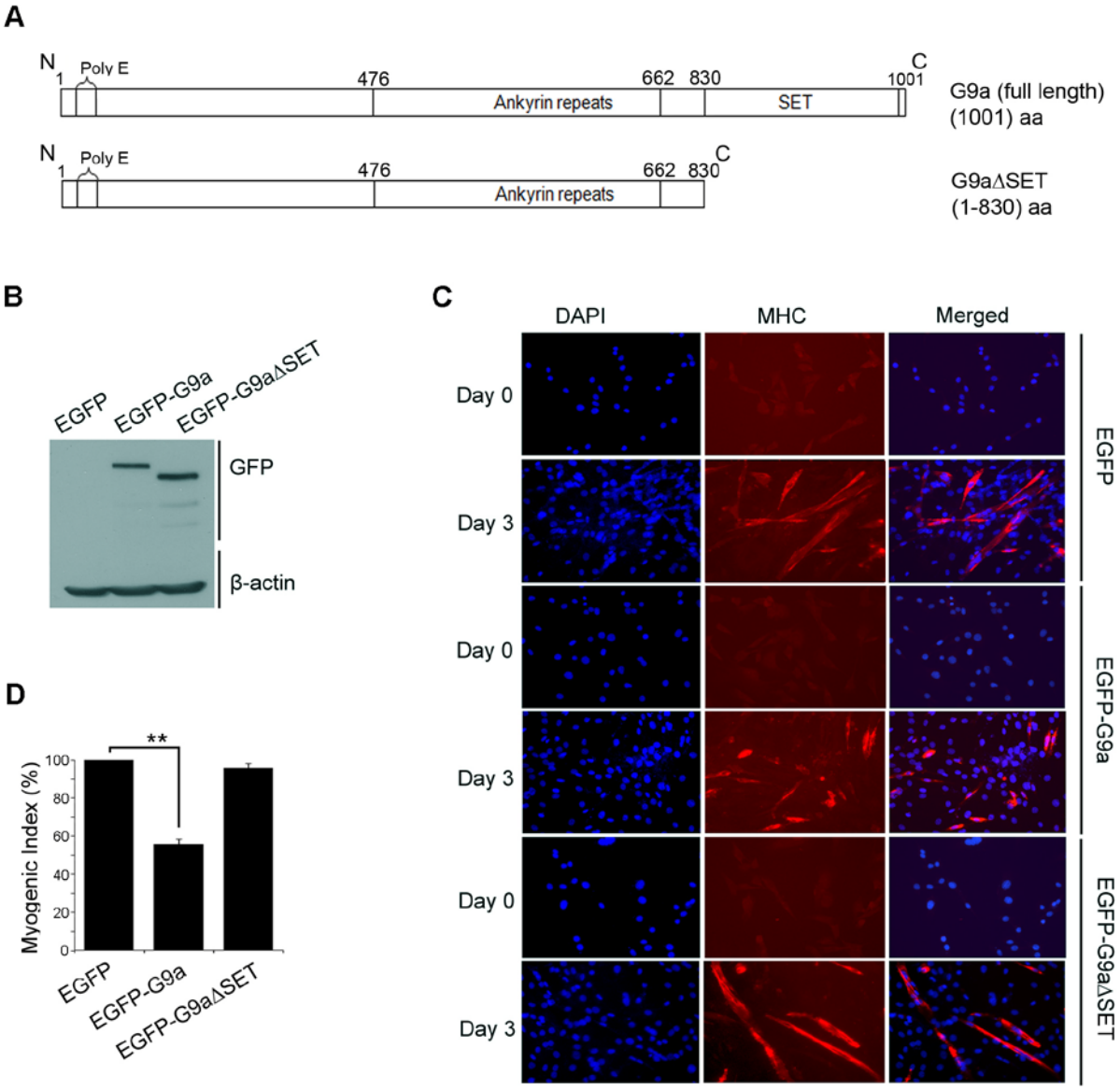

Figure 3.3.3 The SET domain in G9a is required to inhibit differentiation.

A diagram that represents full length G9a (1-1001 aa) and its deletion construct G9aΔSET (1-830 aa). Full length G9a contains 6 contiguous ankyrin repeats and a SET domain. G9aΔSET has its SET domain deleted (A). The G9a expression in EGFP- (vector control), EGFP-G9a- and EGFP-G9aΔSET-expressing cells were analysed by western blot. β-actin was used as a loading control (B). Myotube formation was assessed in undifferentiated cells (day 0) and differentiated cells (day 3) by immunofluorescence staining using anti-MHC antibody (red). Nuclei were stained with DAPI (blue). Images were captured under a microscope using 20x magnification (C). The extent of myogenic differentiation was quantified by myogenic index. Myogenic index was determined in day 3 differentiated cells as the ratio of MHC-

positive stained nuclei to the total nuclei in each field (at least 600 nuclei calculated in each field). Values were presented as means with standard deviation (error bar) and p-values were calculated using Student's t-test (**, $p < 0.01$, highly significant). Results are representative of at least two separate experiments (D).

3.3.4. The SET domain in G9a is required to inhibit expression of muscle-specific genes

In the same set of experiments, cell lysates, collected at undifferentiated day (day 0) and differentiated day (day 1, 3), were subjected to western blot analysis to determine the expression of myogenic differentiation markers, myogenin and troponin T. β-actin was used as an internal control. While both myogenin and troponin T expression was reduced in EGFP-G9a expressing cells, the expression levels in EGFP-G9aΔSET-expressing cells were about the same as that in control cells (Figure 3.3.4). Together, these results demonstrated during G9a-mediated inhibition of muscle differentiation, its methyltransferase activity conferred by its SET domain was necessary for the repression of the muscle genes.

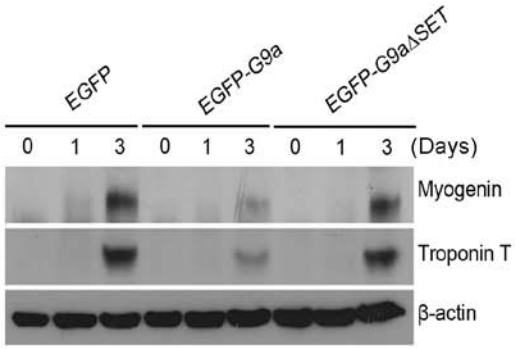

Figure 3.3.4 The SET domain in G9a is required to inhibit expression of muscle-specific genes.

C2C12 cells overexpressing EGFP- (vector control), EGFP-G9a- and EGFP-G9aΔSET-expressing cells were cultured to proliferate (day 0) or induced to differentiate (day 1, 3). Cell lysates were collected and analysed by western blot for

myogenic differentiation markers such as myogenin and troponin T. β-actin expression was used as a loading control.

3.4. G9a methyltransferase inhibitor BIX-01294 induces early onset of differentiation

3.4.1. Titration of BIX-01294 in muscle cells

Previous results showed that G9a was dependent on its methyltransferase activity to block muscle differentiation. G9a methyltransferase activity has been shown to be inhibited by BIX-01294 (a diazepin-quinazolin-amine derivative) (Chang *et al*., 2009, Kubicek *et al.*, 2007). To further determine that G9a-mediated differentiation defect was dependent on its methyltransferase activity, BIX-01294 was used in G9a-overexpressing cells. A range of 0 µM to 6 µM BIX-01294 has been used to block methyltransferase activity in various cell lines (Kubicek *et al*., 2007, Gazzar *et al*., 2008, Chang *et al*., 2009). To determine the optimal BIX-01294 concentration in C2C12 cells that was non-toxic, C2C12 cells cultured in both GM and DM for two days were treated with BIX-01294 ranging from 0 µM to 6 µM, from the beginning in GM. Differentiated cells were stained with MHC antibody. Immunofluorescence analysis showed that C2C12 cells exhibited earlier and enhanced myotube formation with 2.5 µM of BIX-01294 (Figure 3.4.1). At concentration higher than 4 µM BIX-01294 led to cell death. Therefore for subsequent experiments, cells were treated with 2.5 µM BIX-01294.

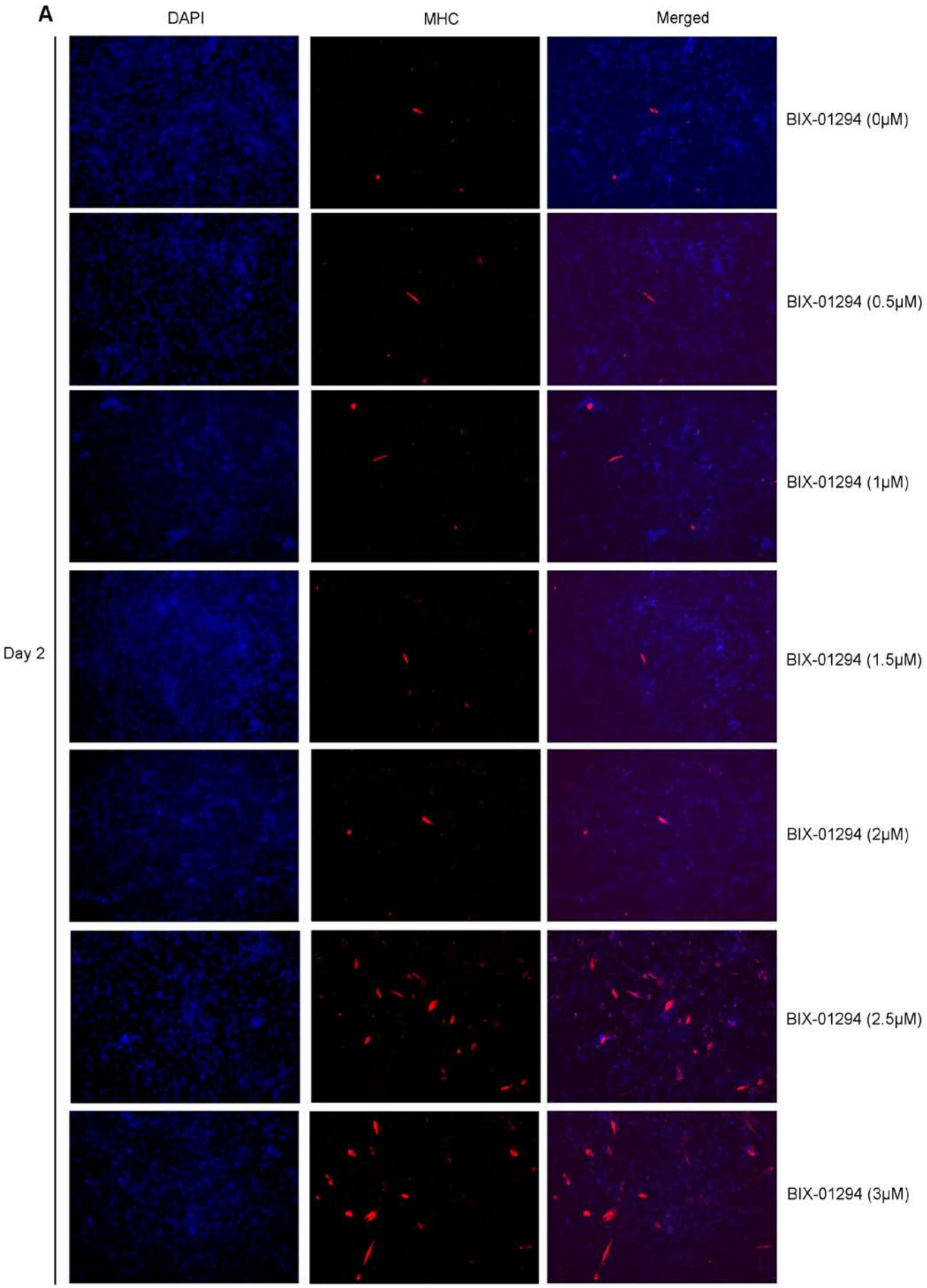
A
DAPI
MHC
Merged
Day 2
BIX-01294 (0μM)
BIX-01294 (0.5μM)
BIX-01294 (1μM)
BIX-01294 (1.5μM)
BIX-01294 (2μM)
BIX-01294 (2.5μM)
BIX-01294 (3μM)

Figure 3.4.1 Titration of BIX-01294 in muscle cells.

C2C12 cells were treated with different concentrations of BIX-01294 and induced to differentiate for two days. Myogenic differentiation was analysed by immunofluorescence staining MHC antibody (red) and nuclei were stained with DAPI (blue). The images were examined under a fluorescence microscope at a 10x magnification.

3.4.2. BIX-01294 induces early onset of differentiation

The extent of differentiation in BIX-01294 treated cells was closely re-examined with more assays performed. C2C12 cells were cultured to induce differentiation for three days and treated with DMSO (control) or 2.5 μM BIX-01294. Differentiated cells were immunostained with MHC antibody and assessed. In comparison with the control-treated cells, BIX-01294-treated cells clearly exhibited more MHC-expressing cells (Figure 3.4.2.A). Therefore, the G9a-inhibitor blocks endogenous G9a methyltransferase activity and induces earlier MHC expression and myotube formation. To assess the extent of the myogenic differentiation induced by BIX-01294, myogenic index was determined. At least 600 nuclei from the immunofluorescence images were counted (Figure 3.4.2.B). BIX-01294 induced a significant increase in myogenic differentiation, with 30% more of MHC-positive cells compared to untreated cells.

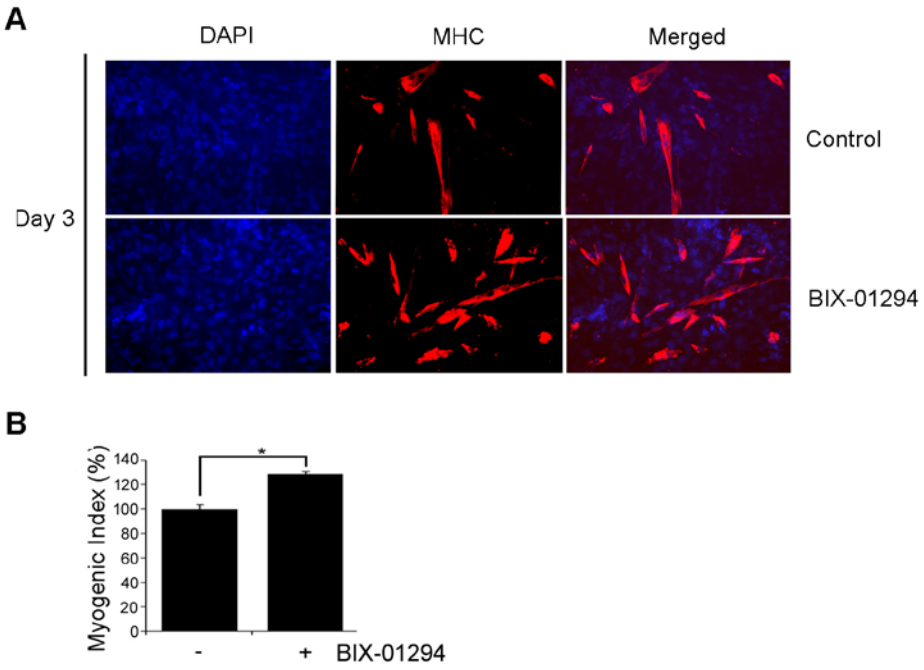

Figure 3.4.2 BIX-01294 induces early onset of differentiation.

C2C12 cells were induced to differentiate for three days and treated with DMSO (control) or 2.5 µM BIX-01294 to block endogenous G9a methyltransferase activity. Myotube formation in the differentiated cells were compared and analysed by immunofluorescence staining. Nuclei were stained with DAPI (blue) and myotube was stained with myosin heavy chain (MHC, red). The images were viewed under a microscope at a 20x magnification (A). The extent of myogenic differentiation was examined by myogenic index. At least 600 nuclei were scored in each field and the values were presented as means with standard deviation (error bar). P-values were determined and shown as * ($p < 0.05$, significant). Results are representative of at least two independent experiments (B).

3.4.3. BIX-01294 induces early expression of muscle-specific genes during differentiation

In addition, cell lysates were collected and analysed by western blot for differentiation markers, myogenin and troponin T. Compared to control cells, in BIX-01294-treated

cells, a higher level of both myogenin and troponin T expression was observed (Figure 3.4.3.A). Similarly, BIX-treated-differentiated cells express higher myogenin mRNA levels than control cells (Figure 3.4.3.B). These data demonstrate that BIX-01294 blocks endogenous G9a methyltransferase activity on myogenic differentiation and induces higher expression of muscle differentiation genes and myotubes formation which leads to earlier onset of differentiation.

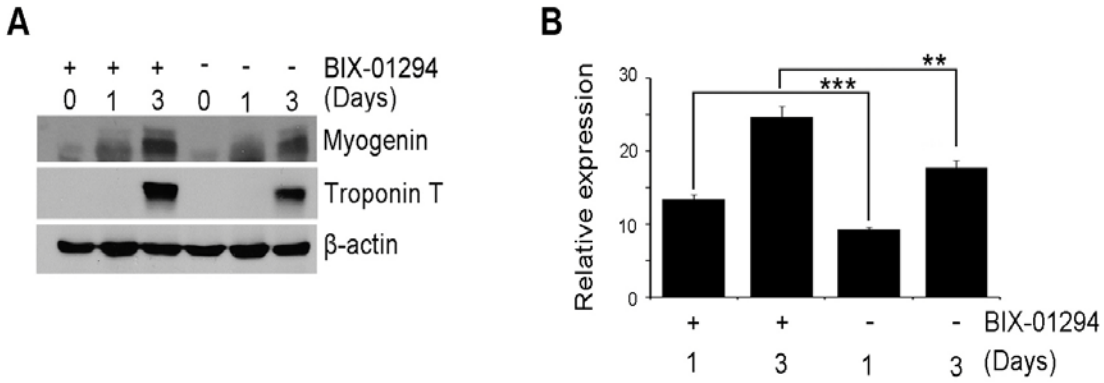

Figure 3.4.3 BIX-01294 induces early expression of muscle-specific genes.
C2C12 cells cultured in GM (day 0) or DM (day 1, 3) were either treated with DMSO (control) or 2.5 μM BIX-01294 to block endogenous G9a methyltransferase activity. Cell lysates at day 0, 1, 3 of cell differentiation were collected and analysed by western blot for differentiation marker myogenin and troponin T. β-actin was used as a loading control (A). Total RNA was extracted from these cells and analysed for myogenin mRNA levels by Q-PCR. These experiments were performed at least twice. Values obtained were standardised against GAPDH (B).

3.4.4. BIX-01294 induces expression of muscle-specific genes in proliferating myoblasts

Myoblasts proliferate in the presence of growth factors and differentiate only upon the absence of growth factors where cells withdraw from the cell cycle. In proliferating

myoblasts, G9a is highly expressed (Figure 3.1.1 - 3.1.2) and overexpression of G9a promotes cell cycle progression (Figure 3.2.1 - 3.2.3) and inhibits muscle differentiation (Figure 3.1.3 - 3.1.4). On the other hand, BIX-01294 induces early onset of differentiation genes (Figure 3.4.2- 3.4.3). To examine if inhibition of G9a can lead to expression of differentiation genes in proliferation conditions, C2C12 cells in proliferating culture were treated with DMSO (control) or 2.5 μM of BIX-01294. Cell lysates were collected and analysed by western blot for early myogenic differentiation marker, myogenin. BIX-01294-treated cells expressed higher levels of myogenin even in the presence of growth factors (Figure 3.4.4.A). Thus, inhibiting endogenous G9a activity in proliferating myoblasts can also induce expression of muscle differentiation marker which is usually not expressed in undifferentiated myoblasts. Consistently, myogenin mRNA levels were significantly increased ($p < 0.001$) and about 1.5 fold higher than in control cells (Figure 3.4.4.B).

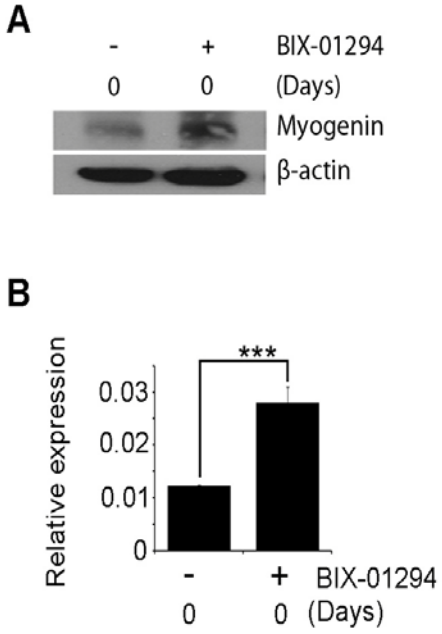

Figure 3.4.4 Inhibition of G9a induces expression of muscle-specific genes in proliferating myoblasts.

C2C12 cells were treated with DMSO or 2.5 μM BIX-01294 in growth medium. Myogenin protein levels were analysed by western blot and standardised to β-actin levels (A). Myogenin mRNA levels were analysed by Q- PCR and standardised to GAPDH. The experiment was done at least twice (B).

3.4.5. BIX-01294 reduces H3K9me2 on myogenin promoter

To examine the impact of BIX-01294 on H3K9me2 on myogenin promoter, C2C12 cells were cultured with DMSO (control) or 2.5 μM of BIX-01294 in GM and then in DM to induce differentiation for two days. Undifferentiated cells (day 0) and differentiated cells (day 2) were harvested and subjected to ChIP assay. In both types of treatment, H3K9me2 levels on the promoter declined as cells differentiated.

Compared to undifferentiated day 0 cells, BIX-01294-treated cells showed a significantly lower (4-fold) H3K9me2 on myogenin promoter. Similarly, upon differentiation, BIX-01294-treated cells showed a significantly lower (2-fold)

H3K9me2 levels on the myogenin promoter (Figure 3.4.5). Thus, BIX-01294 reduces G9a-dependent H3K9 me2 on myogenin promoter in both proliferating and differentiating cells, leading to earlier transcriptional upregulation of myogenic differentiation genes.

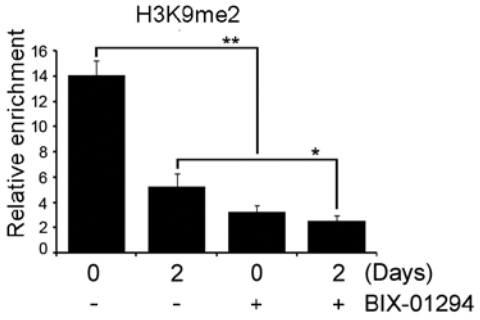

Figure 3.4.5 BIX-01294 decreases H3K9me2 on myogenin promoter.

C2C12 cells were treated with 2.5 μM of BIX-01294 and induced to differentiate for 0, 2 days. ChIP analysis of H3K9me2 on myogenin promoter was assessed. Chromatin was immunoprecipitated with H3K9me2 antibody and analysed by Q-PCR using primers for the myogenin promoter. Primers for β-actin gene were used as an internal control. Values were reported as means with standard deviation (error bar). Results are representative for at least two independent experiments. P-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant), ** ($p < 0.01$, highly significant).

3.5. siRNA knockdown of G9a induces early onset of differentiation

3.5.1. siRNA-mediated knockdown of G9a in human primary myoblasts induces early expression of terminal differentiation genes

It has been shown that siRNA-mediated knockdown of G9a promoted early onset of C2C12 myoblast differentiation (Ling *et al*., 2012). Since C2C12 are immortalized mouse cell line, it was necessary to ensure that the same effect can be achieved in human primary cell cultures. Human primary myoblasts (Lonza) were transfected

with scrambled siRNA control or siRNA targeted to G9a (siG9a). Myoblasts were cultured to proliferate in growth medium or induced to differentiate for three days in differentiating medium. The efficiency of G9a knockdown was determined (Figure 3.5.1.A) and the expression of the myogenic differentiation gene, myogenin and troponin T were also examined by western blotting (Figure 3.5.1.B). siRNA knockdown of G9a in human myoblasts also induced higher expression of both myogenin and troponin T genes that was very similar to the pattern seen using C2C12 myoblasts (Data not found here but shown in Ling *et al*., 2012).

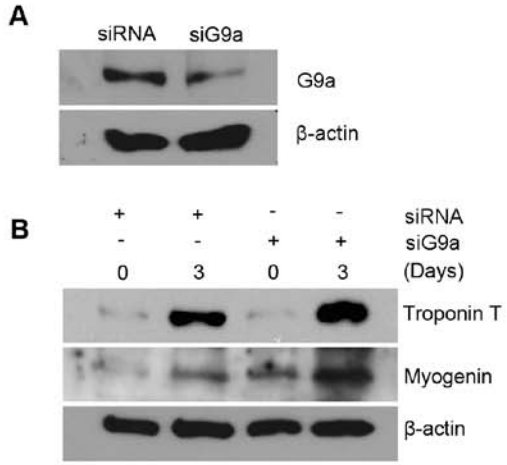

Figure 3.5.1 siRNA-mediated knockdown of G9a in human primary myoblasts induces early expression of differentiation genes.

Human myoblast cells were transfected with scrambled control siRNA or G9a siRNA (siG9a) and induced to proliferate (day 0) or differentiate (day 3). Cell lysates were analysed by western blot for G9a expression (A). Cell lysates were collected for western blot analysis using myogenin and troponin T antibodies (B). β-actin was used as loading control.

3.6. BIX-01294 reverses G9a-mediated inhibition of myogenesis

3.6.1. Inhibition of G9a activity with BIX-01294 rescues differentiation block

To investigate if BIX-01294 can reverse G9a-mediated inhibition of myogenic differentiation, C2C12 cells overexpressing EGFP-G9a were treated with 2.5 μM BIX-01294 or DMSO (control) and induced to differentiate for two days. Differentiated cells were stained with MHC antibody and fluorescence images were analysed. An increase in MHC-positive cells and myotubes were apparent in G9a overexpressing cells treated with 2.5 μM of BIX-01294 than controls (Figure 3.6.1.A). The extent of myogenic differentiation in G9a-expressing cells with control or BIX-01294 treatment was further quantified by myogenic index. The result showed a very significant recovery of myogenic differentiation with BIX-01294 (Figure 3.6.1.B). At the same time, the expression of the muscle differentiation genes was examined in these cells. Cell lysates were collected and subjected to western blot analysis for troponin T. Compared to control G9a-expressing cells, BIX-01294-treated G9a-expressing cells showed improved troponin T expression (Figure 3.6.1.C). Thus, BIX-01294 blocks G9a-mediated inhibition of myogenic genes expression in myogenesis.

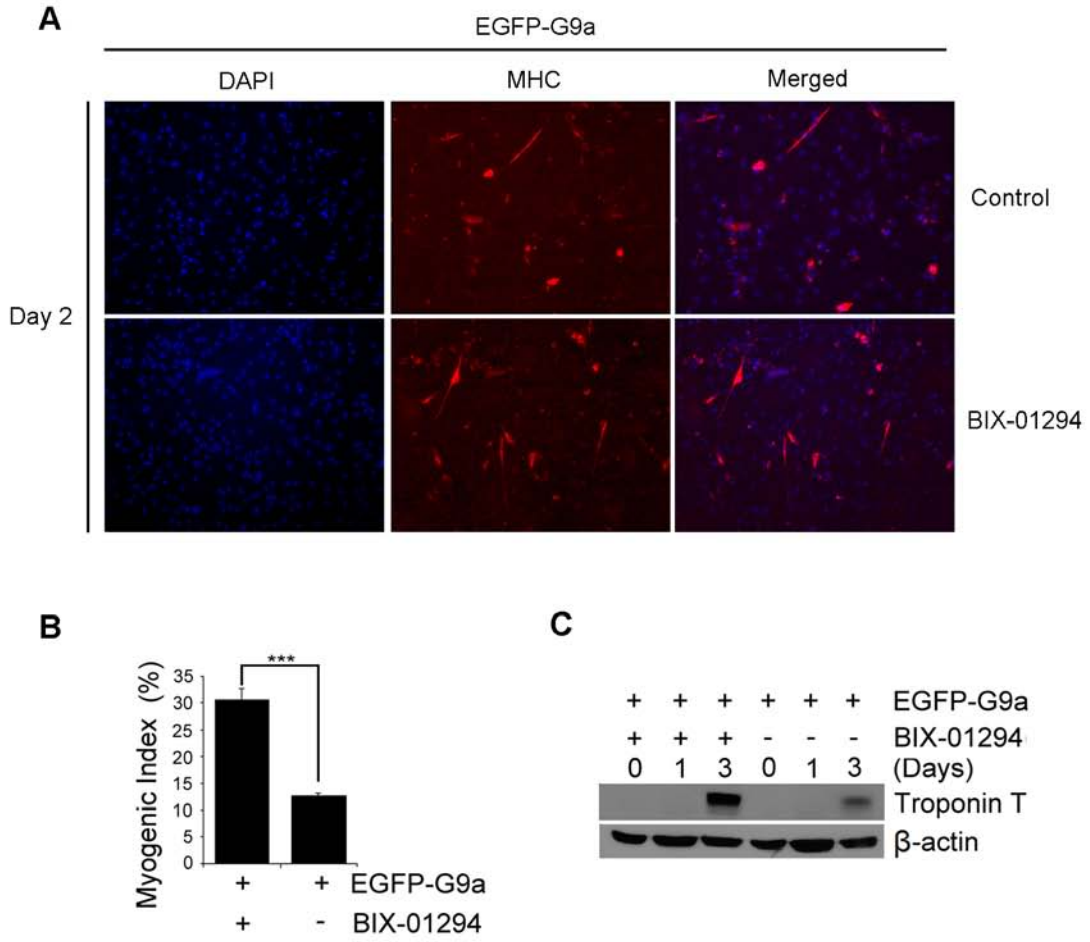

Figure 3.6.1 BIX-01294 blocks G9a-mediated inhibition of myotube formation and muscle-specific genes.

C2C12 cells overexpressing EGFP-G9a were treated with DMSO (control) or 2.5 µM of BIX-01294 and induced to differentiate for two days. Myogenic differentiation was analysed by immunofluorescence staining using myosin heavy chain (MHC, red) antibody. Nuclei were stained with DAPI (blue). The images were examined under a fluorescence microscope at a 10x magnification (A). The extent of myogenic differentiation was assessed in EGFP-G9a-expressing cells, treated with DMSO (control) or 2.5 µM BIX-01294. Myogenic index, determined from at least 600 nuclei, was presented as means with standard deviation (error bar). These experiments were conducted at least twice. P-values were calculated and indicated as *** ($p < 0.001$, highly significant). (B). Cell lysates from day 0, 1, 3 of differentiated cells were analysed by western blot for troponin T. β-actin was used as a loading control (C).

3.7. Re-expression of MyoD rescues G9a-mediated inhibition of myogenesis

3.7.1. MyoD(K014R) is more effective in rescuing the differentiation defect imposed by G9a compared to wild type MyoD

Apart from H3K9, G9a has also been recognized for its ability to methylate non-histone proteins such as CDYL1, WIZ, ACINUS and C/EBPβ (Rathert *et al*., 2008). Both the human and mouse MyoD cDNA sequence also displayed a similar G9a-methylation consensus motif RK at K104. To test if G9a-mediated repression of differentiation is reversible and whether re-expression of MyoD can rescue G9a-mediated differentiation defect, differentiation assays were performed. Since MyoD activity can be inhibited by G9a methylation, a mutant MyoD(K104R) (with point mutation of 104 lysine to arginine) was also tested. C2C12 cells were transduced with pBabe-G9a and selected with puromycin for two days. Puromycin-selected cells expressing G9a were then transfected with pCS2 (vector control), MyoD and MyoD(K104R) mutant. A day post-transfection, cells were induced to differentiate for three days. Cell lysates were collected and analysed by western blot. Transfected G9a, wild type and MyoD(K104R) were expressed in cells (Figure 3.7.1.A). Differentiation was monitored by MHC staining. Re-expression of MyoD partially restored differentiation in G9a overexpressing cells and the rescue was better with equivalent amounts of MyoD(K104R) mutant (Figure 3.7.1.B). This indicated that MyoD can rescue differentiation defects but since MyoD may still be methylated and kept inactive by G9a, MyoD(K104R) mutant can rescue differentiation to a greater extent.

The extent of the myogenic differentiation was assessed. Both MyoD and MyoD(K104R) were able to rescue the differentiation block imposed by G9a, with MyoD(K104R) showing a significantly stronger rescue (Figure 3.7.1.C). Similar

rescue experiment was performed using CH310T1/2 cells with identical results obtained (Data not found here but shown in Ling *et al*., 2012).

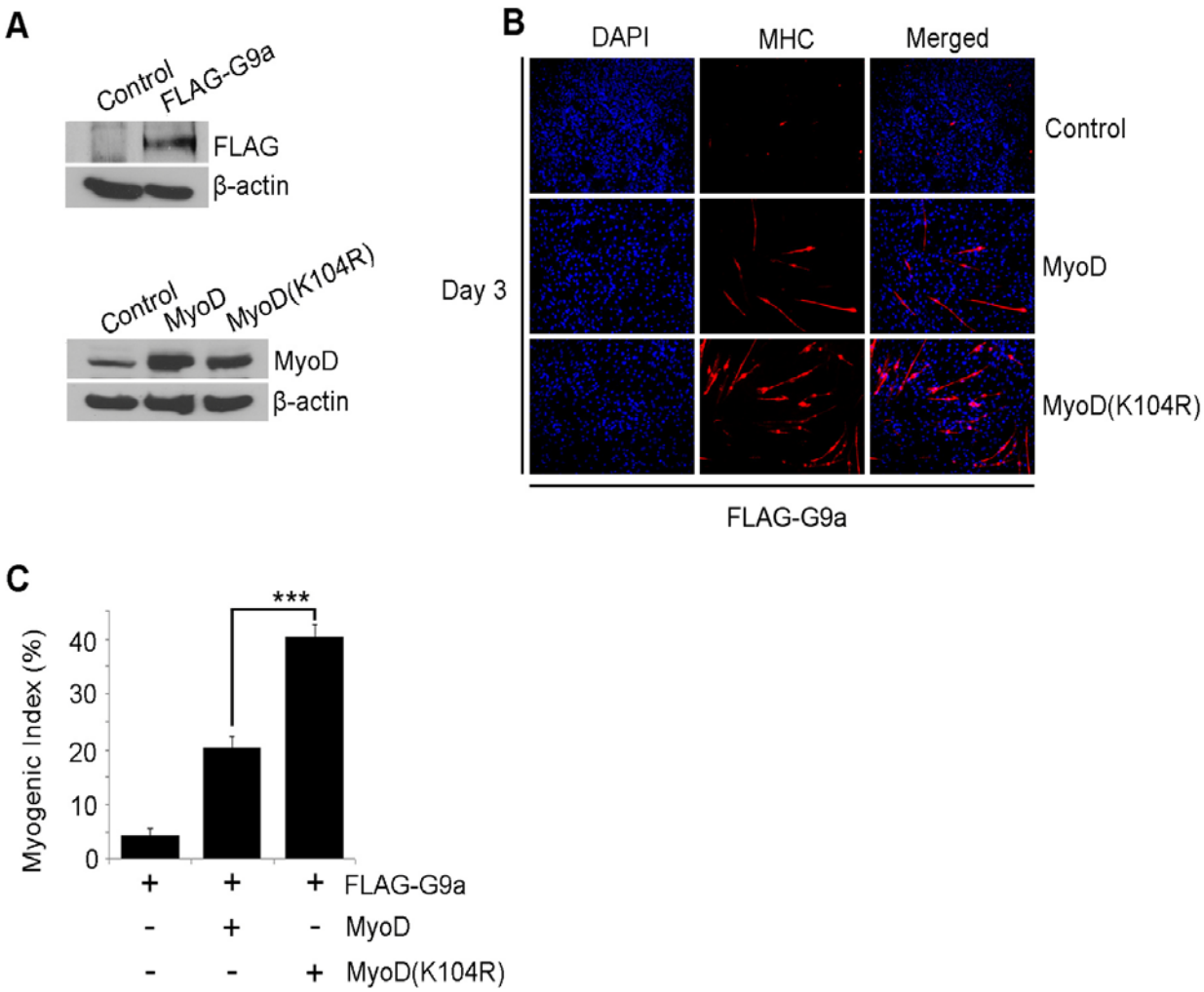

Figure 3.7.1 MyoD(K104R) is more effective in rescuing the differentiation defect imposed by G9a compared to wild type MyoD.

FLAG-G9a-expressing C2C12 cells were transfected with pCS2 (control), MyoD and MyoD(K104R) and induced to differentiate for three days. G9a expression in cells was determined by western blot (A, upper panel). MyoD expression in cells overexpressing FLAG-G9a with pCS2(control), MyoD or MyoD(K104R) was determined by western blot. β-actin act as a loading control (A, lower panel). Myogenic differentiation was monitored by immunofluorescence staining using anti-

myosin heavy chain (MHC, red) antibody. Nuclei were stained with DAPI (blue). Images were captured under a microscope with 10x magnification (B). Myogenic index was determined from at least 600 nuclei and presented as means with standard deviation (error bar). P-values were calculated using Student's t-test and shown as *** ($p<0.001$, highly significant). The experiments were performed at least twice with comparable results (C).

3.7.2. Re-expression of MyoD(K104R) in G9a overexpressing cells rescues MyoD target gene expression to a higher level than MyoD

To examine skeletal muscle protein expression, both undifferentiated (day 0) and differentiated (day 2) cell lysates were collected and analysed by western blot for troponin T expression. Troponin T was expressed to a higher level in cells re-expressing MyoD(K104R) compared to MyoD (Figure 3.7.2). Together, these data suggested that the G9a is capable of methylating and inhibiting MyoD from activating muscle gene expression. Increasing MyoD levels reverses the inhibitory effect of G9a on myogenic differentiation.

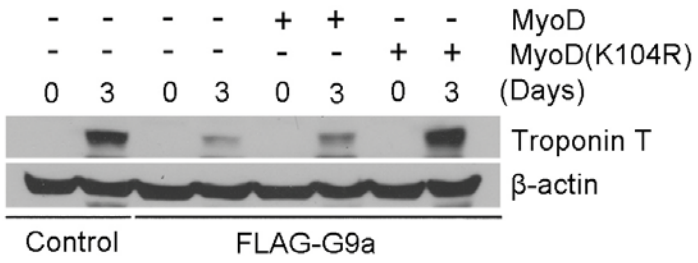

Figure 3.7.2 Re-expression of MyoD(K104R) in G9a overexpressing cells rescues MyoD target gene expression to a higher level than MyoD.

FLAG-G9a-expressing C2C12 cells were transfected with pCS2 (control), MyoD and MyoD(K104R) and induced to differentiate for three days. The terminal

differentiation gene troponin T expression was assessed in cells by western blot. β-actin was used as a loading control.

3.8. Sharp-1 inhibits myogenic differentiation

Together, earlier results indicate that G9a has an inhibitory role in myogenic differentiation. As G9a is a transcriptional co-repressor that needs to associate with transcription factors to inhibit muscle gene transcription, it is necessary to identify the transcription factor which recruits G9a to the myogenin promoter. On the other hand, Sharp-1 has been identified as a transcription repressor in both myogenesis and adipogenesis. While inhibiting adipogenic differentiation, Sharp-1 recruits G9a to the adipogenic promoter. However, Sharp-1 mechanism in inhibiting myogenic differentiation is not well-defined and it not known if Sharp-1 also recruits G9a to regulate myogenesis. Therefore, there is a need to determine the association between Sharp-1 and G9a and if G9a is involved in Sharp-1-mediated repression.

3.8.1. Sharp-1 expression declines upon myogenic differentiation

It is important to ensure uniformity and consistency with published data and hence the pilot experiment is to confirm that the decline in Sharp-1 expression levels during myoblast differentiation is observed. Cells were cultured to proliferate and then induced to differentiate for one to three days. Cell lysates were collected at the respective time points and Sharp-1 mRNA levels were reversed transcribed and analysed by Q-RT-PCR using primers for mSharp-1 and GAPDH (primer sequences are shown in table II). The relative Sharp-1 mRNA expression values obtained were normalised against GAPDH mRNA levels which serve as an internal control. Sharp-1 mRNA expression declined during muscle cells differentiation (Figure 3.8.1.). This

result is similar to our previous study which showed that Sharp-1 downregulated during myogenic differentiation (Azmi *et al*., 2004).

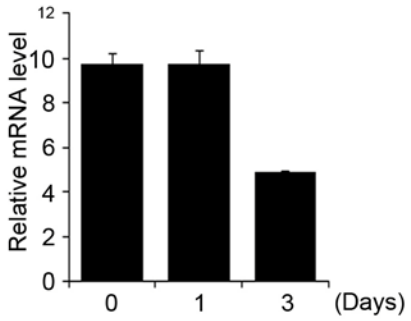

Figure 3.8.1 Expression of Sharp-1 in C2C12 myoblasts.

Sharp-1 mRNA levels were analysed in C2C12 cells cultured in GM (day 0) or induced to differentiate for one to three days (day 1, 3). Total RNA was isolated and Sharp-1 mRNA levels were analysed by RT-PCR and Q-PCR as described in materials and methods. Quantified relative amount of Sharp-1 mRNA were normalised to house-keeping gene GAPDH and presented in graph as mean with standard deviation (error bar). Results are representative of at least two separate experiments.

3.8.2. Overexpression of Sharp-1 inhibits differentiation

The expression of Sharp-1 declines as myoblasts differentiate and studies have shown that overexpression of Sharp-1 inhibits myogenic differentiation (Azmi *et al*., 2004). Similar differentiation assay was conducted to confirm consistency with previous report. C2C12 cells were co-transfected with pCS2 (vector control) or Sharp-1 and pBabe-puro at a ratio of 9:1. After selection with puromycin, cells were used for induced to differentiate for three days. The expression of Sharp-1 and loading control β-actin were determined by western blot (Figure 3.8.2.A). The myotubes in control or

Sharp-1-expressing C2C12 cells were immunostained MHC antibody. More MHC-expressing myotubes were observed in control cells than in Sharp-1-expressing cells (Figure 3.8.2.B). The extent of the myotube formation was quantified using myogenic index with at least 600 nuclei counted. Compared to control cells, there was a significance ($p < 0.01$) reduction in level of MHC expressed in Sharp-1-expressing cells (Figure 3.8.2.C). Hence, the result is in line with previously reported data (Azmi *et al*., 2004, Fujimoto *et al*., 2007).

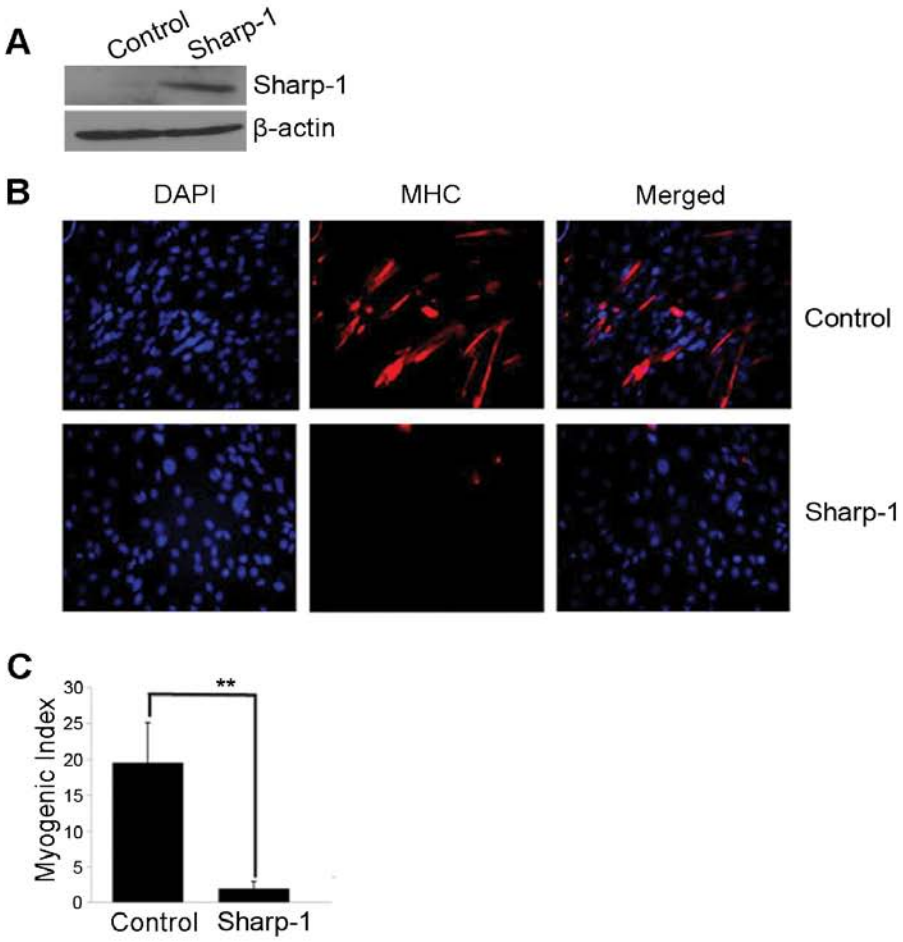

Figure 3.8.2 Overexpression of Sharp-1 inhibits myoblast differentiation.
C2C12 cells were transfected with pCS2 (control) or Myc-Sharp-1 and pBabe-puro at a ratio of 9:1. After selection with puromycin for two days, transfection-positive cells were cultured to proliferate in growth medium (day 0) or induced to differentiate in differentiating medium for one to three days (day 1, 3). The expression of Sharp-1

was determined using western blot analysis and β-actin was used as a loading control (A). Day 3 differentiated cells were immunostained for terminal differentiation marker myosin heavy chain (MHC) primary antibody and Texas-red secondary antibody. Nuclei were counterstained with DAPI (blue) (B). The extent of myotube formation was determined using myogenic index. The values were reported in the graph are means with standard deviation (error bars) from at least three separate experiments. The significance level are presented, $p<0.01$ (C).

3.8.3. Overexpression of Sharp-1 inhibits expression of MyoD target genes

The expression of myogenin and troponin T were examined in the total cell lysates collected from proliferating cells (day 0) and differentiating cells (day 1, 3). In addition to immunofluorescence analysis of MHC, biochemical analysis of terminal differentiation markers showed that myogenin and troponin T were reduced in C2C12 cells overexpressing Sharp-1 (Figure 3.8.3). These morphological and biochemical results are similar to those reported previously in literature. Thus, Sharp-1 inhibits expression of the myogenic differentiation markers and impairs skeletal muscle differentiation.

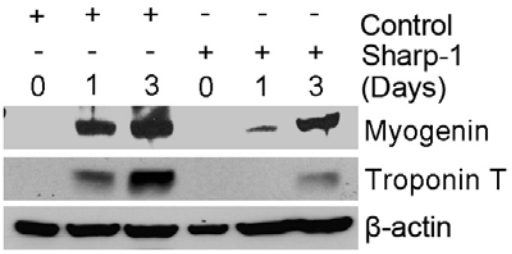

Figure 3.8.3 Sharp-1 inhibits expression of MyoD target genes.

Cell lysates were collected from day 0, 1 and 3 of control- and Sharp1-expressing cells and analysed for myogenin and troponin T expressions by western blot.

3.9. Sharp-1 associates with G9a

3.9.1. G9a deletion mutant constructs

Since Sharp-1 inhibits myogenic differentiation (Azmi *et al.*, 2004) and G9a on its own also inhibits myogenesis, I next tested the possibility that Sharp-1 associates with G9a to inhibit muscle gene expression and myogenic differentiation. G9a contains an ankyrin repeats and a catalytic SET domain. To define the domain in G9a required for association with Sharp-1, full length G9a was used along with deletion mutant lacking the ankyrin repeats (G9aΔANK) or both ankyrin repeats and the SET domain (G9aΔANKSET). A schematic diagram of the constructs is shown below (Figure 3.9.1).

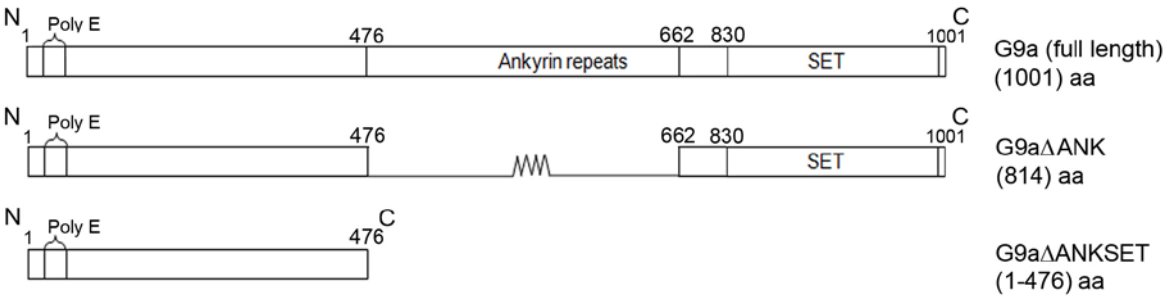

Figure 3.9.1 A schematic representation of G9a and its deletion mutants.

A diagram that represents full length G9a (1-1001 aa) and truncated constructs G9aΔANK (814 aa) and G9aΔANKSET (1-476 aa). Full length G9a contains 6 contiguous ankyrin repeats and a SET domain. G9aΔANK has its ankyrin repeats domain deleted and G9aΔANKSET has both ankyrin repeats domain and SET domain deleted.

3.9.2. Sharp-1 associates with G9a through its ankyrin repeats

While C2C12 mouse myoblast is the most appropriate cell line to use for interaction study, it is also a difficult-to-transfect cell line. Nonetheless, the important interaction study using endogenous G9a and Sharp-1 in myoblasts was also performed and shown in Figure 3.9.7. Here, instead of C2C12, a common highly-transfectable adherent cell lines such as 293T cells were cotransfected with Myc-Sharp-1 and full length FLAG-G9a or FLAG-G9aΔANKSET mutant. The expression of FLAG-G9a, FLAG-G9aΔANKSET and Myc-Sharp-1 was determined by western blot analysis. Cell lysates were immunoprecipitated with anti-c-Myc-agarose beads and immunoprecipitates were analysed by western blot with anti-FLAG antibody. FLAG-G9a, FLAG-G9aΔANKSET and Myc-Sharp-1 were expressed in cells. Immunoblot analysis showed that only full length FLAG-G9a interacted with Sharp-1 (Figure 3.9.2.A). This indicated that the ANK and/or SET domain of G9a is necessary for association with Sharp-1. Similar immunoprecipitation assays were performed with FLAG-G9a, FLAG-G9aΔANK and Myc-Sharp-1. Deletion of the ankyrin domain in G9a abrogated its interaction with Sharp-1 (Figure 3.9.2.B). These demonstrated that Sharp-1 associates with G9a through its ankyrin repeats.

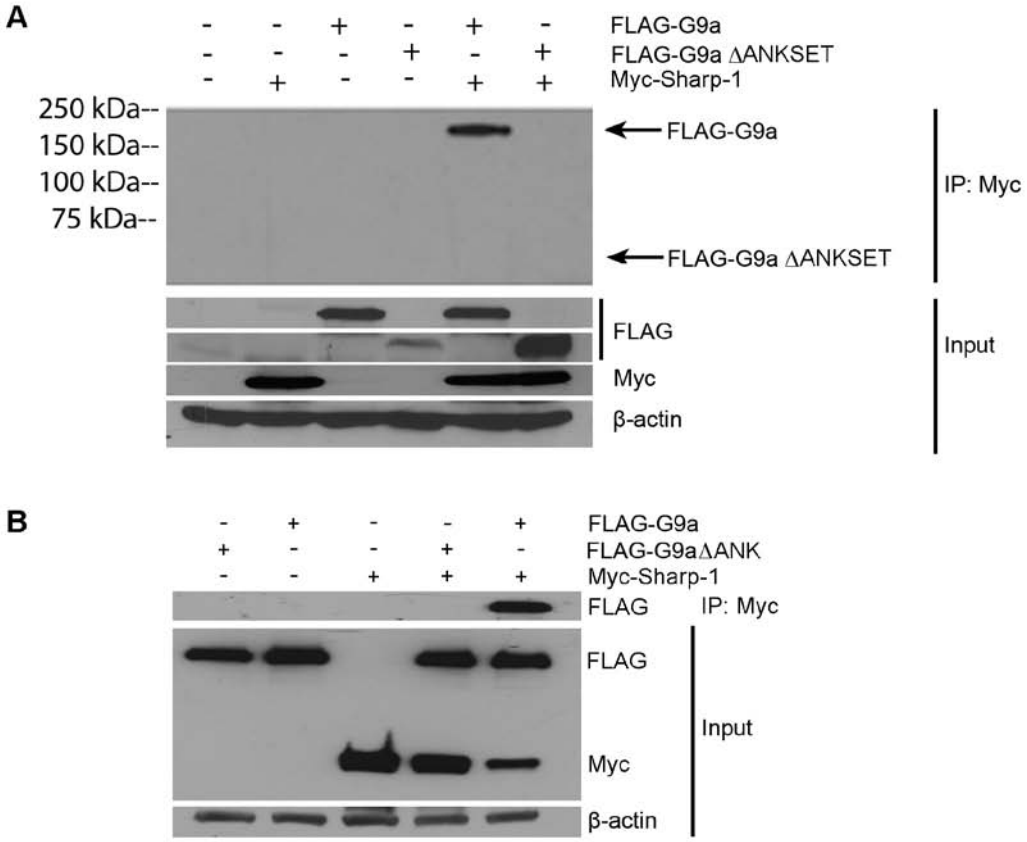

Figure 3.9.2 Sharp-1 associates with G9a through its ankyrin repeats.

A diagram that represents full length G9a (1-1001 aa) contains 6 contiguous ankyrin repeats and a SET domain. G9aΔ ANK (814 aa) with ankyrin repeats domain deleted and G9aΔANKSET (1 -476 aa) with both ankyrin repeats domain and SET domain deleted) (A). 293T cells were co-transfected with Myc-Sharp-1, FLAG-G9a or, FLAG-G9aΔANKSET (A) or FLAG-G9a with FLAG-G9aΔANK (B). Cell lysates and immunoprecipitates were anaylzed by western blot with anti-FLAG and anti-c-Myc antibodies. β-actin was used as an internal loading control.

3.9.3. Sharp-1 deletion mutant constructs

Sharp-1 contains a basic region, a helix-loop-helix (HLH) domain, an orange (O) domain and Glycine/Alanine rich C-terminus. Several deletion constructs were generated which are shown schematically (Figure 3.9.3). Sharp-1-bHLH mutant

contains only the N-terminus with basic and HLH domain; Sharp-1ΔO mutant and Sharp-1ΔC mutant have their orange domain or C-terminus truncated respectively. Sharp-1ΔO mutant and Sharp-1ΔC mutant were generated by PCR using primers and annealing temperatures as shown in table IV and recloned into BamHI/AscI sites and EcoRI/ApaI sites of pCS-Myc-Sharp-1 plasmid.

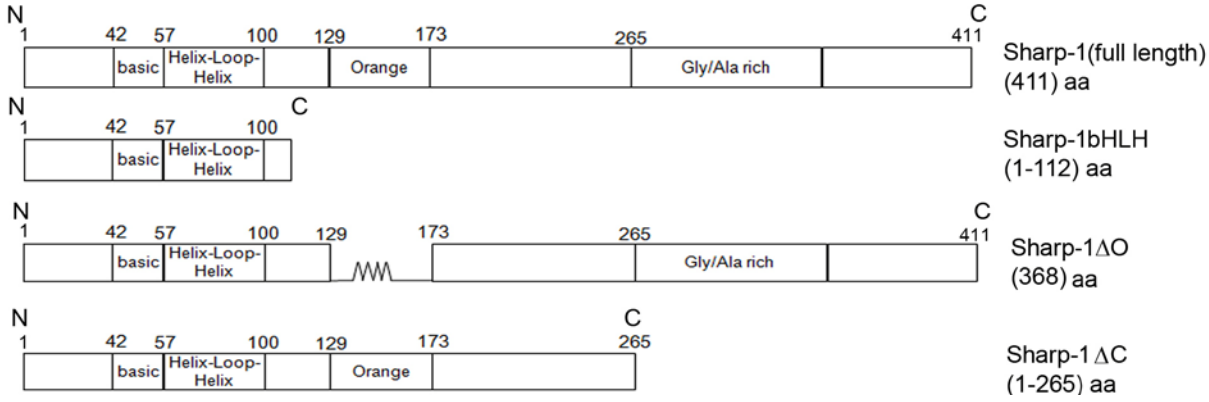

Figure 3.9.3 A schematic diagram of Sharp-1 and its deletion mutants.

A diagram that represents full length Sharp-1 (1-411 aa) and its truncated construct Sharp-1 bHLH (1-112 aa), Sharp-1 ΔO (368 aa), and Sharp-1 ΔN (1-265 aa). Sharp-1 contains a DNA binding basic domain, helix-loop-helix (HLH) domain, an orange domain and a Glycine/Alanine rich region. Sharp-1 bHLH consists of only the N-terminus with basic domain and HLH domain. Sharp-1 ΔO has its orange domain deleted and Sharp-1 ΔC has its C-terminus deleted as indicated.

3.9.4. G9a associates with Sharp-1 through its C-terminus spanning amino acid 173 to 265

Immunofluoresence studies were carried out to determine the subcellular localisation of each protein. C2C12 cells were co-transfected with FLAG-G9a and Myc-Sharp-1 or its mutants and detected with G9a and anti-c-Myc antibodies. G9a, Sharp-1 and all

its mutants demonstrated punctuate staining and remained localised in the nucleus (Figure 3.9.4.A). To identify the region in Sharp-1 that binds to G9a, immunoprecipitation assay was performed using G9a, full length Sharp-1 and its mutants. Similar to figure 3.9.2, instead of C2C12, highly transfectable 293T cells were co-transfected with FLAG-G9a and Myc-Sharp-1 or its mutants. Cell lysates were subjected to immunoprecipitation with anti-c-Myc agarose beads and western blot analysis. Immunoprecipitates demonstrated that all Sharp-1 constructs except Myc-Sharp-1-bHLH interacted with G9a (Figure 3.9.4.B) and the region spanning amino acid 173 to 265 in Sharp-1 were important in the interaction with G9a.

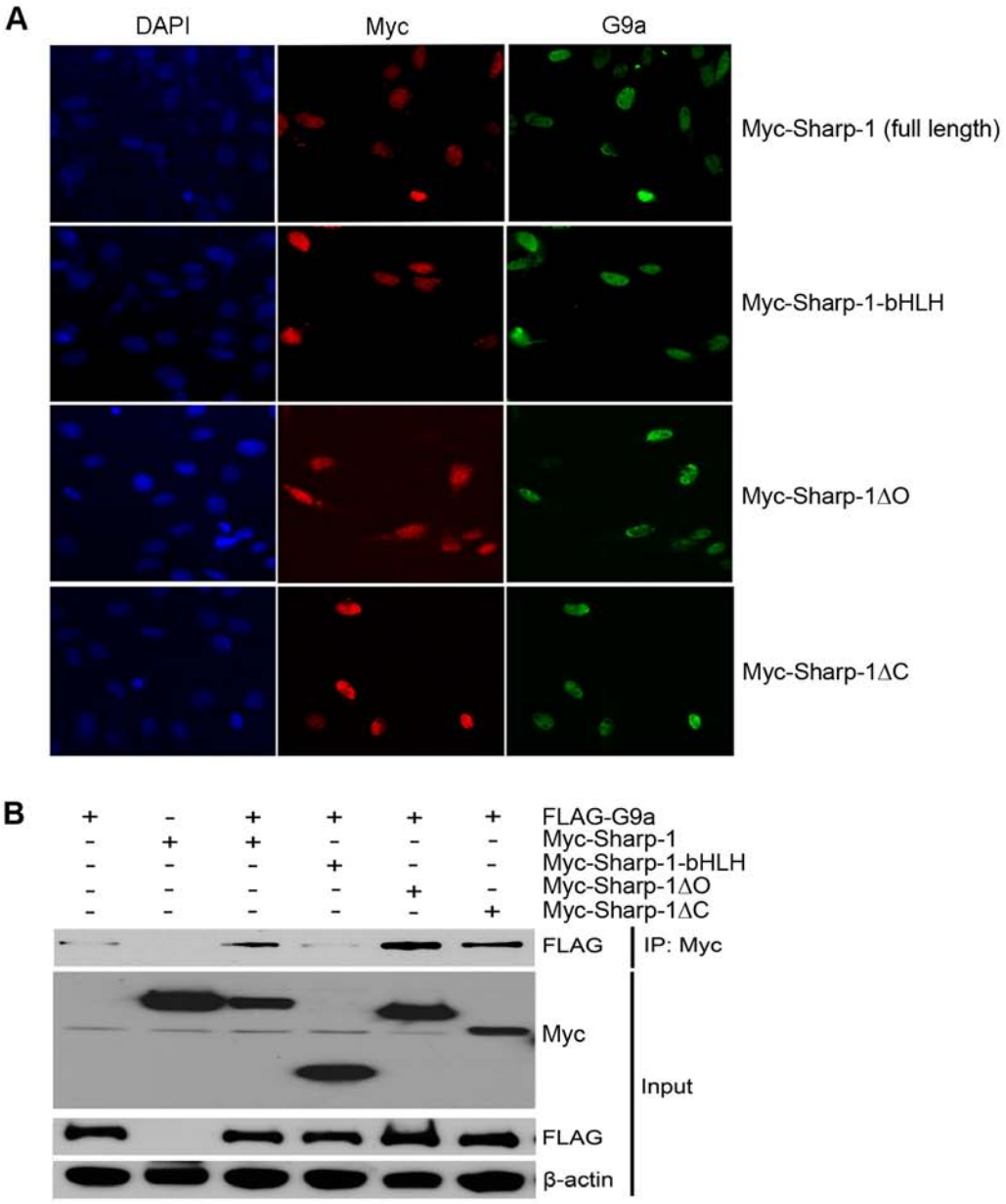

Figure 3.9.4 Localisation of Sharp-1 mutants.

Immunofluorescence staining showing nuclear staining of Myc-Sharp-1, its mutants (red) and FLAG-G9a (green) in C2C12 cells. Nuclei were stained with DAPI (blue) (A). 293T cells were co-transfected with FLAG-G9a and Myc-Sharp-1 or its mutants as shown. Whole cell lysates were immunoprecipitated with anti-c-Myc agarose beads. Cell lysates and immunoprecipitates were analysed by western blot with anti-FLAG, anti-c-Myc and β-actin antibodies (B).

3.9.5. G9a co-localises with Sharp-1 in the nucleus

Sharp-1 associates with G9a and to ensure that they co-exist in the nucleus, co-localisation of G9a and Sharp-1 was examined. Mammalian cells such as COS 7 cells, NIH3T3 cells, C3H10T½ cells and C2C12 cells were co-transfected with FLAG-G9a and Myc-Sharp-1. The cells were immunostained with G9a and Myc antibodies and the nuclei were stained with DAPI. The fluorescence images were merged to determine co-localisation as indicated by the yellow fluorescence. Both G9a and Sharp-1 are localised in the nucleus in all types of cells indicating that their association is not cell type specific (Figure 3.9.5).

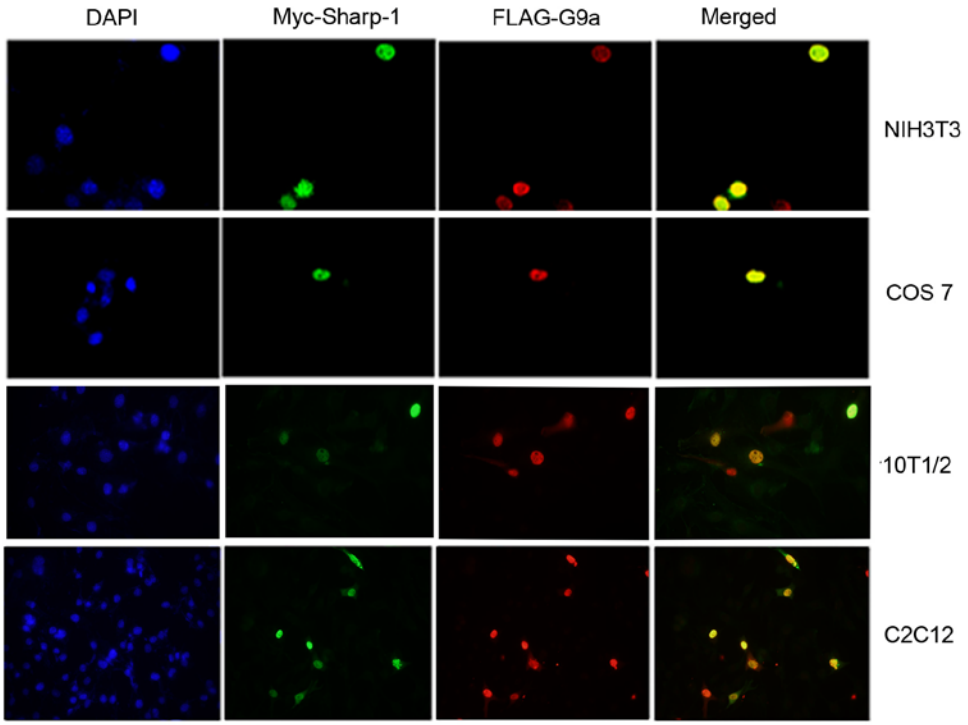

Figure 3.9.5 G9a co-localises with Sharp-1 in the nucleus.

NIH3T3, COS7, C3H10T½ and C2C12 cells were co-transfected with Myc-Sharp-1 and FLAG-G9a. Co-localisation of Sharp-1 and G9a was analysed by immunofluorescence staining using anti-c-Myc antibody (green) and anti-G9a antibody (red). Nuclei were stained with DAPI (blue). The images were merged and

co-localisation of these proteins was visualized by yellow fluorescence. Images were captured under a microscope with 20x magnification.

3.9.6. G9a interacts directly with Sharp-1

G9a associates with Sharp-1 and to further determine whether interaction between the two proteins is direct, a glutathione S-transferase (GST) pull down assay was performed. GST and GST-Sharp-1 were induced with isopropyl-beta-D-thiogalactopyranoside in *E.coli* BL21(DE3) cultures. GST proteins were purified using Glutathione Sepharose 4B beads and detected by coomassie blue staining on SDS-PAGE gel. The amount of GST protein (indicated as **) and GST-Sharp-1 protein (indicated as *) were quantified (Figure 3.9.6.A). FLAG-G9a was transcribed and translated *in vitro* using TNT coupled reticulocyte lysate system (Promega). The same amount (10 µg) of GST and GST-Sharp-1 proteins were incubated with FLAG-G9a, followed by glutathione-sepharose beads. Proteins immobilized on glutathione-sepharose beads were subjected to western blot analysis with anti-FLAG antibody. 10% of *in vitro* translated G9a protein was loaded as a control. GST-Sharp-1, but not GST, interacted with FLAG-G9a (Figure 3.9.6.B), demonstrating that G9a interacts directly

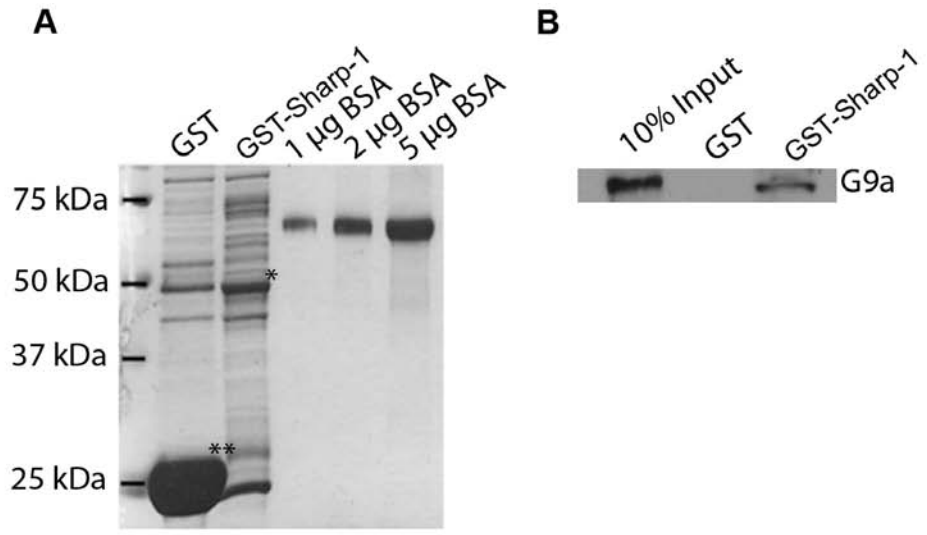

Figure 3.9.6 G9a interacts directly with Sharp-1.

Recombinant GST (**) and GST-Sharp-1 (*) fusion proteins expressed in *E.coli* were detected by coomassie blue staining (A). *In vitro* translated FLAG-G9a was incubated with GST or GST-Sharp-1 fusion proteins to determine its direct protein interaction. 10% of FLAG-G9a used in pull-down assay was used as input and detected by western blot with anti-G9a antibody (B).

3.9.7. G9a interacts with Sharp-1 in myoblasts

The association of G9a and Sharp-1 was clear. Earlier studies showed that the proteins were expressed in the nucleus in both proliferating myoblasts and differentiated myotubes. In addition, an immunoprecipitation study using 293T cells showed that they interact. However, 293T cells are non-muscle cells. Hence, the following immunoprecipitation assay was required to ensure that if they also interact in myoblasts undergoing proliferation and differentiation. As there was some technical difficulty with antibody in detecting endogenous Sharp-1, C2C12 cells were transfected with Myc-Sharp-1. Cells were induced to proliferate in GM or induced to differentiate for two days in DM. Both undifferentiated and differentiated cell lysates were collected and analysed by western blot for the expression of endogenous G9a and overexpression of Myc-Sharp-1. Cell lysates were subjected to immunoprecipitation with anti-G9a antibody, followed by protein A/G agarose beads. Subsequently, immunoprecipitates were analysed by western blot with anti-c-Myc antibody. The figure showed that G9a interact with Sharp-1 in both the proliferative myoblasts and differentiated myoblasts (Figure 3.9.7.). The result revealed that G9a and Sharp-1 interacted in the proliferating myoblasts and although their expression decline, their interactions remained during the cells differentiation.

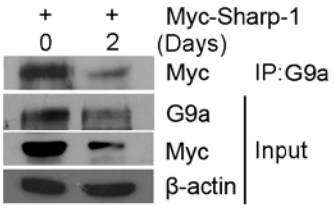

Figure 3.9.7 Endogenous G9a interacts with Sharp-1 in myoblasts.

C2C12 cells were transfected with Myc-Sharp-1 and induced to differentiate for two days. Cell lysates were immunoprecipitated with anti-c-Myc agarose beads. Cell lysates and immunoprecipitates were analysed by western blot with anti-c Myc, anti-G9a antibodies. β-actin served as an internal control.

3.9.8. Sharp-1 co-localises with di-methylated histone 3 lysine 9

Since Sharp-1 associates with G9a, I examined if Sharp-1 co-localises with di-methylated histone H3K9. C2C12 cells were immunostained with Sharp-1/DEC2 and H3K9me2 antibodies, followed by secondary fluorescence FITC and Texas-red antibodies. Nuclei were stained with DAPI. Fluorescence images showed that Sharp-1 co-localised with di-methylated histone H3K9 in the nucleus of myoblasts (Figure 3.9.8.).

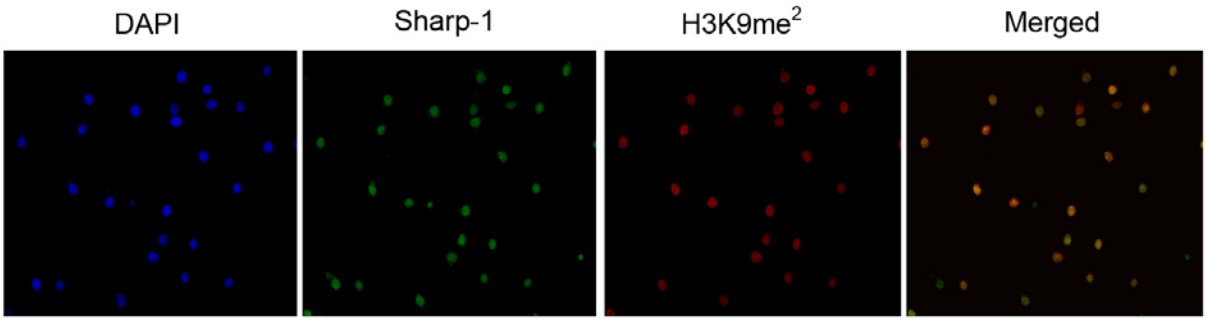

Figure 3.9.8 Sharp-1 co-localises with di-methylated histone 3 lysine 9.
Co-localisation of Sharp-1 and H3K9me2 was examined by immunofluorescence staining. C2C12 cells were immunostained with DEC2/ Sharp-1 antibody (green) and H3K9me2 antibody (red). Nuclei were stained with DAPI (blue). Images were merged and co-localised proteins were visualized by yellow fluorescence.

3.10. Sharp-1 recruits G9a to block myogenic transcriptional activity

3.10.1. G9a enhances Sharp-1-mediated inhibition of E-box-dependent transcriptional activity

To examine the role of G9a in Sharp-1-dependent transcriptional repression, luciferase reporter assays were carried out. C2C12 cells were transfected with 6E-box driven firefly luciferase reporter (6E-TATA-Luc) with increasing amount of Sharp-1. Cell lysates were collected after 24 hours for luciferase activity analysis using Dual-reporter luciferase assay system kit (Promega). The data showed that the transcriptional activity of the reporter was significantly inhibited with increasing amount of Sharp-1 (Figure 3.10.1.A). To examine the role of G9a, C2C12 cells were transfected with 6E-box driven firefly luciferase reporter (6E-TATA-Luc), Sharp-1 and increasing amount of G9a. After 24 hours, renilla and luciferase activities were measured from the harvested cells. Sharp-1 transcriptional repression activity was enhanced with increasing amount of G9a (Figure 3.10.1.B). Thus, Sharp-1-mediated inhibition of transcriptional activity is augmented with methyltransferase G9a. In addition, a similar luciferase reporter assay was done to show that the transcriptional activity can be suppressed further by increasing both the transcriptional repressor Sharp-1 and transcriptional co-repressor G9a (Figure 3.10.1.C).

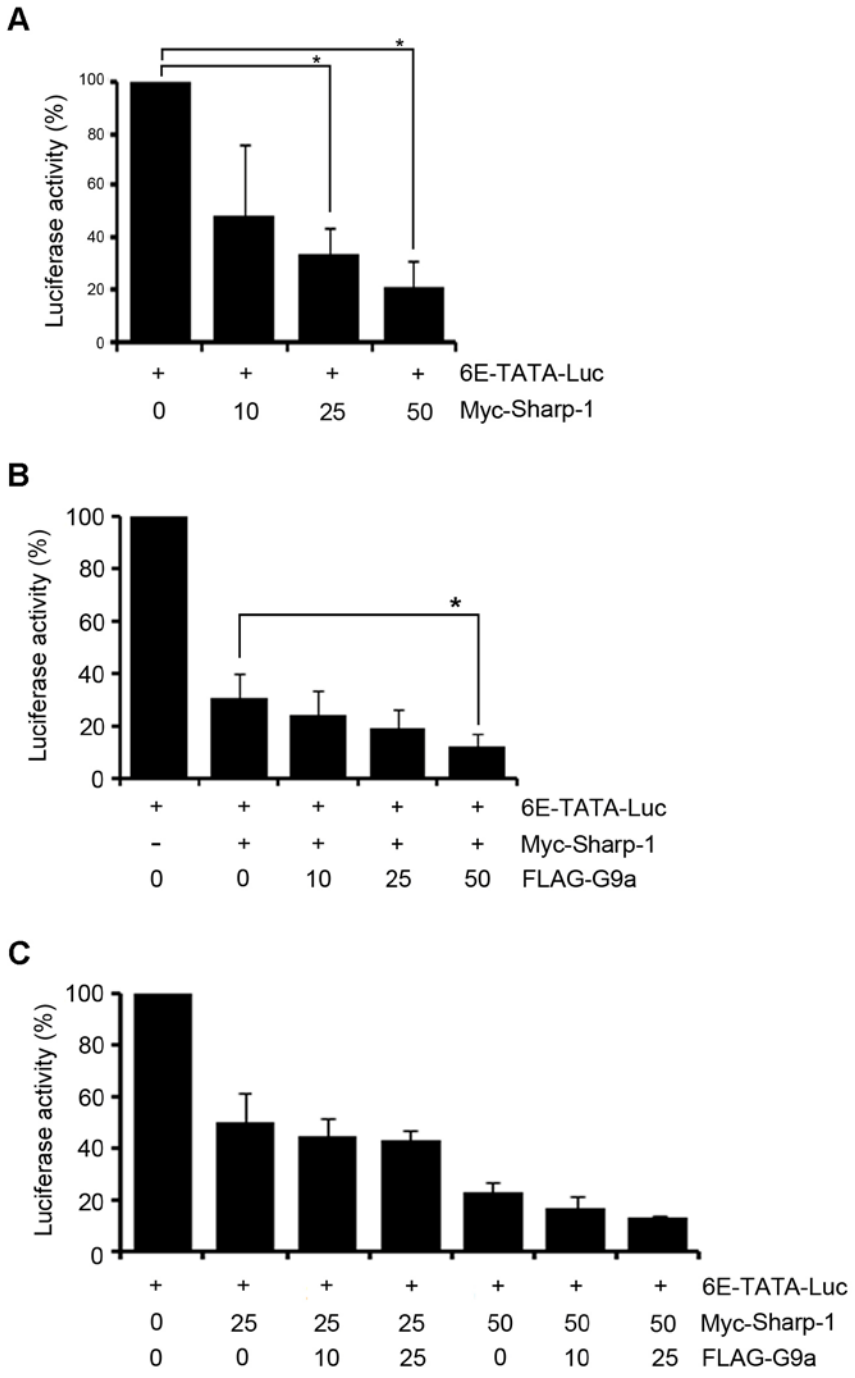

Figure 3.10.1 G9a enhances Sharp-1 repression activity.

C2C12 cells were transfected with 6E-box driven firefly luciferase reporter (6E-TATA-Luc, 100 ng) with increasing amounts of Myc-Sharp-1 (ng)(A) or Myc-Sharp-1 (50 ng) and with increasing amount of FLAG-G9a (ng) (B) or Sharp1 (25ng and 50ng) with increasing amount of FLAG-G9a (ng) (C). After 24 hours, luciferase

activity was measured and normalised to renilla (2.5ng) luciferase. The mean value with standard deviation (error bar) was presented as percentage of the control (6E-TATA-Luc only). P-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant). These experiments were performed at least twice.

3.10.2. G9a enhances Sharp-1-mediated repression of MyoD transcriptional activity

G9a enhances Sharp-1-mediated repression (Figure 3.10.1.) and we examined its impact on Sharp-1-dependent MyoD activation of myogenin promoter. To examine MyoD transcriptional activity, it is necessary to use a cell line such as C3H10T½ fibroblast cells which does not express endogenous MyoD. In reporter assay, exogenous expression of MyoD will bind to myogenin promoter-driven firefly luciferase reporter (pMyogLuc which contain myogenin promoter sequence) and drive the transcription of the luciferase reporter gene, thus giving a high luciferase activity. Hence, cells were transfected with pMyogLuc, MyoD, Myc-Sharp-1 and FLAG-G9a. After 24 hours, renilla and luciferase activities were measured. MyoD activated myogenin transcription was given a value of 100%, MyoD transcriptional activity was inhibited by Sharp-1 (48%) or G9a (45%) alone. In addition, this inhibitory effect was greater in the presence of both G9a and Sharp-1 (21%) (Figure 3.10.2.). Thus, G9a co-operates with Sharp-1 in the transcriptional repression of MyoD activity and activation of the myogenin promoter.

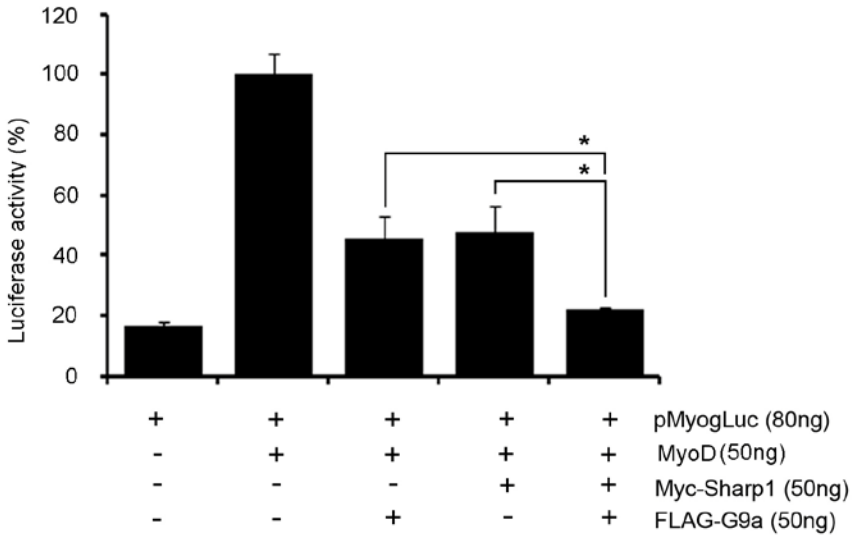

Figure 3.10.2 G9a enhances Sharp1-mediated inhibition of MyoD transcriptional activity.

C2C12 cells were transfected with myogenin promoter-drivenfirefly luciferase reporter (pMyogLuc), MyoD, Myc-Sharp1 and FLAG-G9a. 24 hours after transfection, luciferase activity was measured and normalised to Renilla luciferase. The mean value with standard deviation (error bar) was presented as percentage of control (80ng of MyogLuc with 50ng of MyoD). The assay was repeated at least twice with similar results. P-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant).

3.10.3. G9aΔANK does not impact Sharp-1-dependent repression of the myogenin promoter and Sharp-1-bHLH-dependent repression is not increased by G9a

To examine if the interaction of G9a and Sharp-1 is essential for repression of myogenin expression, C3H10T½ fibroblast cells were transfected with myogenin promoter-driven firefly luciferase reporter (pMyogLuc), MyoD, Myc-Sharp-1, Myc-Sharp-1-bHLH, FLAG-G9a and FLAG-G9aΔANK as indicated (Figure 3.10.3.). In

contrast to full length Sharp-1 which significantly inhibited MyoD activity, Sharp-1bHLH repressed MyoD to a lesser extent and repression was not significantly increased in presence of G9a. Conversely, while G9a enhanced Sharp-1-dependent repression of MyoD, it did not have a major impact on repression by Sharp-1bHLH. These data support the notion Sharp-1-mediated repression of MyoD-transcriptional activity is partially dependant on recruitment of G9a.

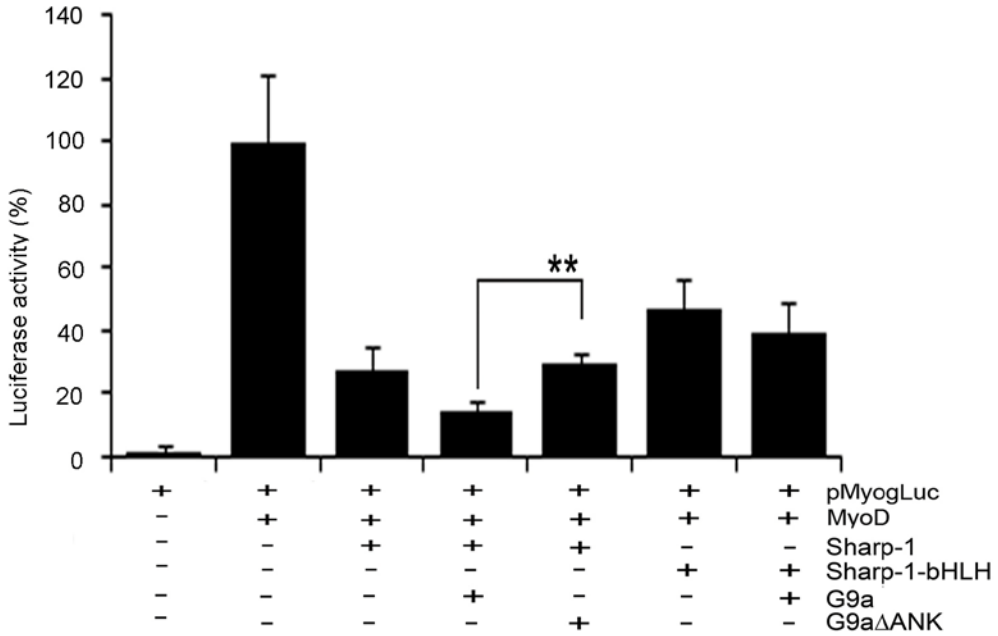

Figure 3.10.3 G9aΔANK does not impact Sharp-1-dependent repression of the myogenin promoter and Sharp-1-bHLH-dependent repression is not increased by G9a.

C2C12 cells were transfected with myogenin promoter-driven firefly luciferase reporter (pMyogLuc, 80 ng), MyoD, Myc-Sharp1, Myc-Sharp-1-bHLH, FLAG-G9a and FLAG-G9aΔANK (50 ng). 24 hours after transfection, luciferase activity was measured and normalised to renilla (2.5 ng) luciferase. The mean value with standard deviation (error bar) was presented as percentage of control (MyogLuc with MyoD).

The assay was performed at least two times with comparable results. P-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant).

3.11. Sharp-1 overexpression is associated with enhanced G9a-mediated H3K9me2 on myogenin promoter

3.11.1. Sharp-1 enhances H3K9me2 on myogenin promoter

Both the transcription factor Sharp-1 and transcriptional co-factor methyltransferase G9a inhibit skeletal muscle differentiation (Azmi *et al.*, 2004, Figure 3.1.). In addition, Sharp-1 co-localises with both G9a and di-methylated histones H3K9 and interacts with methyltransferase G9a in the nucleus (Figure 3.9.). Since, G9a mediates repressive histone methylation marks, I investigated whether G9a-mediated H3K9me2 was apparent in presence of Sharp-1 on the myogenin promoter. The H3K9me2 levels on the myogenin promoter was assessed using ChIP assay. C2C12 cells were infected with pBabe (vector control) and pBabe-Sharp-1 retrovirus and expression of Sharp-1 was determined with DEC2 (Sharp-1) antibody (Figure 3.11.1.A). Cells were cultured to proliferate in GM or DM for two days. Cells were harvested and subjected to ChIP assay with H3K9me2 antibody. Cells overexpressing Sharp-1 showed a similiar H3K9me2 pattern to that of cells overexpressing G9a (Figure 3.3.1.). Compared with control cells, H3K9me2 in Sharp-1-expressing cells was significantly higher on the myogenin promoter in both undifferentiated and differentiated cells (Figure 3.11.1.B).

Moreover, in the control cells, an increase of H3K9K14ac level on myogenin promoter correlated with an increase in the expression of muscle genes required during cells differentiation. In contrast, the H3K9K14ac level was reduced in Sharp-1-expressing cells (Figure 3.11.1.C). Thus, elevated Sharp-1 expression enhances

H3K9me2 and reduces H3K9K14ac level at the myogenic promoter which correlates with reduced gene transcription and reduced the expression of myogenic genes.

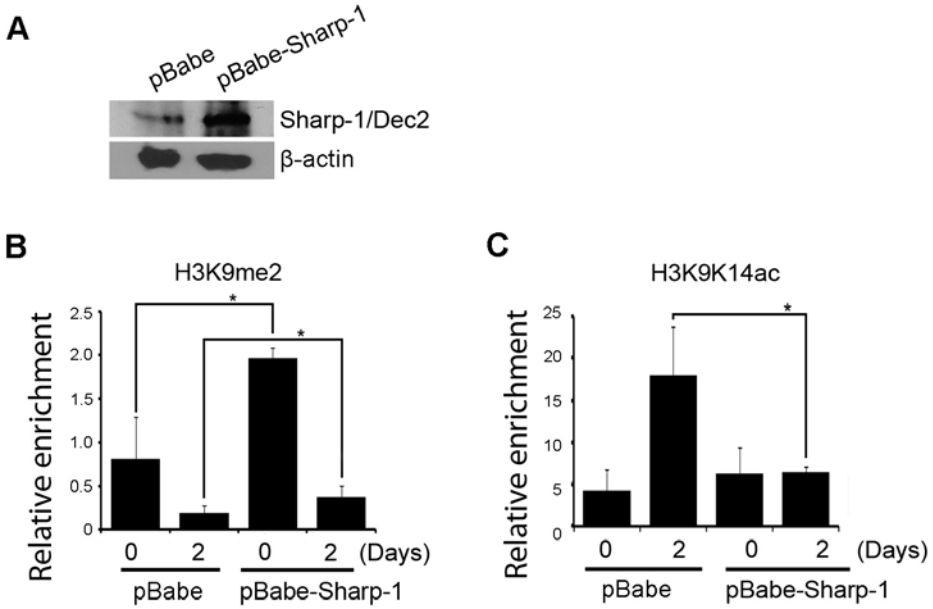

Figure 3.11.1 Overexpression of Sharp-1 alters H3K9me2 and H3K9K14ac levels on myogenin promoter.

Sharp-1 expression in Phoenix cells was analysed by western blot using DEC2 (Sharp-1) antibody. β-actin was used as a loading control (A). C2C12 cells were infected with pBabe (vector control) or pBabe-Sharp-1 retrovirus. Transfected cells were selected with puromycin and induced to proliferate (day 0) or differentiate (day 2). Cells were fixed and subjected to ChIP analysis using H3K9me2 antibody (B) or H3K9K14ac antibody (C). Immunoprecipitated chromatin was analysed by Q-PCR using primers for myogenin promoter and normalised to internal control ß-actin gene. Values are presented as means with standard deviation (error bar). Results are representative of at least two independent ChIP experiments. P-values were calculated using Student's t-test and indicated as * ($p < 0.05$, significant).

3.12. Sharp-1 enhances G9a-mediated methylation of MyoD

3.12.1. Sharp-1 associates with G9a to methylate MyoD at lysine K104

Earlier it was shown that MyoD(K104R) is more effective that wild-type MyoD in rescuing the differentiation defect which indicates that methylation of MyoD at Lysine 104 was crucial in G9a-mediated inhibition of myogenic differentiation. Hence, besides investigating that Sharp-1 associates with G9a-mediated methylation of H3K9 on the myogenin promoter, it is also necessary to investigate if methylation of MyoD also occurs in Sharp-1-expressing cells. C3H10T½ mouse fibroblast cells were used to further examine the role of Sharp-1 in association with G9a in myogenesis. C3H10T½ mouse fibroblast cells do not express MyoD and can be converted to myoblast-like cells upon transfection with MyoD (Salvatori *et al*., 1995). Cells were transfected with FLAG-G9a, Myc-Sharp-1, MyoD and MyoD(K104R) mutant. Cell lysates were immunoprecipitated with MyoD and subjected to western blot analysis. The expression of FLAG-G9a, MyoD, MyoD(K104R) mutant, Myc-Sharp-1 was determined. In the presence of Sharp-1, higher levels of methylated MyoD were apparent. However, G9a was not able to methylate MyoD(K104R) mutant in the absence or presence of Sharp-1 (Figure 3.12.1.). These studies suggest that Sharp-1, G9a and MyoD may co-exist in protein complex and Sharp-1 enhances methylation of MyoD at lysine site 104.

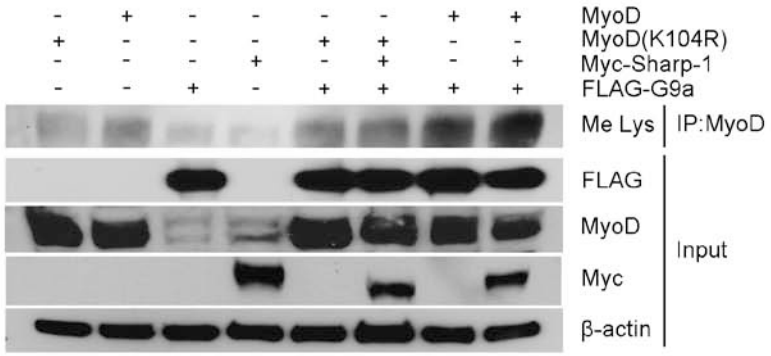

Figure 3.12.1 Sharp-1 enhances MyoD methylation at lysine K104.

C3H10T½ cells were transfected with MyoD, MyoD(K104R), FLAG-G9a and Myc-Sharp-1 and immunoprecipitated with MyoD antibody. The expression of G9a, MyoD and MyoD(K104R) and Sharp-1 in cell lysates was analysed by western blot with anti-FLAG antibody, anti-c-Myc antibody, anti-MyoD antibody. The MyoD immunoprecipitates were analysed by western blot with methyl lysine (Me Lys) antibodies.

3.12.2. Sharp-1 represses MyoD transcriptional activity to a greater extent than MyoD(K104R)

Sharp-1 is known to block MyoD transcriptional activity partially through its bHLH domain but this does not appear to be dependent on the recruitment of HDAC1 (Fujimoto *et al*., 2007). In the presence of Sharp-1, both increased MyoD methylation at K104 and a higher level of H3K9me2 was observed. To examine the impact of Sharp-1 on the transcriptional activity of MyoD and MyoD(K104R), a luciferase reporter assay was performed. C3H10T½ fibroblast cells were transfected with pMyogLuc, Sharp-1, MyoD and MyoD(K104R) and cultured in differentiating medium. After 24 hours, cell lysates were collected and luciferase and renilla activities were measured. Sharp-1 repressed both MyoD and MyoD(K104R) transcriptional activity but the repression of MyoD was greater compared to the repression of MyoD(K104R) (Figure 3.12.2.). This indicates that Sharp-1-mediated inhibition of MyoD transcriptional activity involves methylation of MyoD at K104.

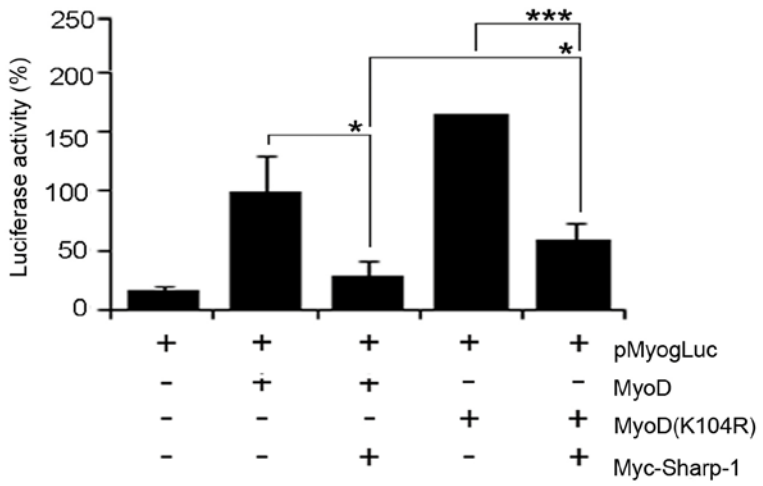

Figure 3.12.2 Sharp-1 represses MyoD transcriptional activity to a greater extent than MyoD(K104R).

C3H10T½ cells were transfected with myogenin promoter-driven firefly luciferase reporter (pMyogLuc)(80 ng), MyoD, MyoD(K104R) and Myc-Sharp-1 (50 ng). Cells were cultured in differentiation medium. After 24 hours, luciferase activity was measured and normalised to renilla luciferase (2.5 ng). The mean value with standard deviation (error bar) was presented as percentage of control (pMyogLuc and MyoD). The assay was repeated at least twice. P-values were obtained using Student's t-test and shown as * ($p < 0.05$), *** ($p<0.001$).

3.13. BIX-01294 rescues Sharp-1-mediated inhibition of myogenesis

3.13.1. Inhibition of G9a activity reverses Sharp-1-mediated repression

G9a enhanced Sharp-1-mediated repression of E-box-dependent transcriptional activity. To confirm that this Sharp-1-mediated repression was associated with G9a methyltransferase activity, we performed a similar luciferase assay with the G9a inhibitor, BIX-01294. C2C12 cells were transfected with 6E-box driven firefly luciferase reporter, Sharp-1 and incubated with 2.5 µM of BIX-01294. In presence of

BIX-01294, Sharp-1 transcriptional repression was ameliorated (Figure 3.13.1.A). Moreover, treatment with BIX-01294 significantly improves MyoD transcriptional activity that was repressed by Sharp-1 (Figure 3.13.1.B) confirming that G9a methyltransferase activity is involved in Sharp-1-mediated repression of MyoD activity. C3H10T½ fibroblast cells were transfected with myogenin promoter-driven firefly luciferase reporter (pMyogLuc), MyoD, Myc-Sharp-1 and incubated with 2.5 µM of BIX-01294

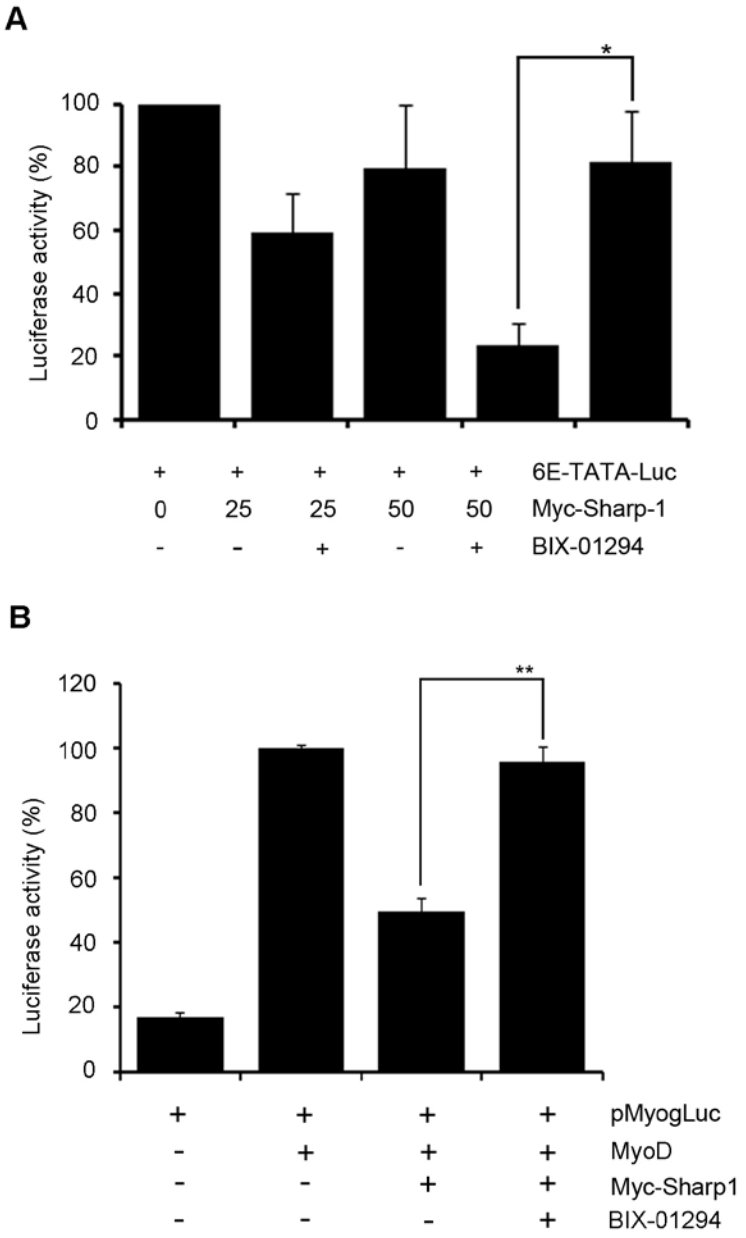

Figure 3.13.1 BIX-01294 rescues Sharp-1-mediated inhibition of transcriptional activity.

C2C12 cells were transfected with 6E-box driven firefly luciferase reporter (6E-TATA-Luc, 100 ng), Myc-Sharp-1 and in the presence or absence of 2.5 µM BIX-01294 (a G9a inhibitor)(A). C3H10T½ cells were transfected with myogenin promoter-driven firefly luciferase reporter (pMyogLuc, 80 ng), MyoD (50 ng), Myc-Sharp-1 (50 ng) and incubated with 2.5 µM of BIX-01294 (B). 24 hours after transfection, luciferase activity was measured and normalised to renilla (2.5 ng)

luciferase. The mean value with standard deviation (error bar) was presented as percentage of the control (6E-TATA-Luc or pMyogLuc). Values are representative of at least two independent experiments. P-values were calculated using Student's t-test and shown as * ($p < 0.05$, significant) and ** ($p < 0.01$, very significant)

3.13.2. BIX-01294 reverses Sharp-1-mediated H3K9me2 on myogenin promoter

BIX-01294 blocks Sharp-1 repression of MyoD activity. To examine its impact on Sharp-1-induced H3K9me2 on myogenin promoter, ChIP assays were carried out in Sharp-1-overexpressing cells without and with BIX-01294 treatment. C2C12 cells were infected with pBabe-Sharp-1 retrovirus, and Sharp-1 expression in cells was confirmed by western blot analysis (Figure 3.13.2.A). One set of cells expressing Sharp-1 was treated with 2.5 μM of BIX-01294 while the other set was treated with same volume of DMSO (control). Cells, cultured in GM and then induced to differentiate in DM for two days, were subjected to ChIP assays with H3K9me2 antibody. Both DMSO-treated and BIX-01294-treated cells showed a decrease in the H3K9me2 on myogenin promoter as cell differentiated. However, compared to DMSO-treated Sharp-1 expressing cells, treatment with BIX-01294 resulted in lower H3K9me2 on myogenin promoter (Figure 3.13.2.B). Therefore, the G9a inhibitor BIX-01294 inhibits Sharp-1-mediated repression of MyoD activity and reduces H3K9me2 on myogenin promoter.

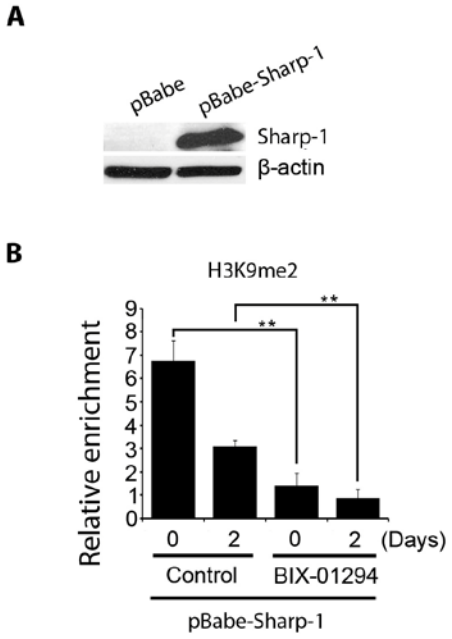

Figure 3.13.2 BIX-01294 rescues Sharp-1-induced H3K9me2 on myogenin promoter.

Sharp-1 expression in Phoenix cells was determined by western blot analysis. β-actin was used as a loading control (A). C2C12 cells were infected with pBabe-Sharp-1 retrovirus. Cells were treated with DMSO (control) or 2.5 μM of BIX-01294 and induced to differentiate for two days. Cell lysates were subjected to ChIP analysis using H3K9me2 antibody. DNA was analysed by Q-PCR using primers for the myogenin promoter and the values obtained were normalised to β-actin. Relative DNA level was determined and presented as means with standard deviation (error bar). Similar results were observed in at least two separate experiments. p-values were calculated using Student's t-test and indicated as ** ($p < 0.01$, very significant) (B).

3.13.3. BIX-01294 reduces Sharp-1-mediated methylation of MyoD

Sharp-1 associates with G9a and mediates H3K9me2 on myogenin promoter. Moreover, Sharp-1 represses MyoD transcriptional activity during the inhibition of skeletal muscle genes (Azmi *et al.*, 2004, Figure 3.9.-3.11.). Since overexpression of

G9a inhibit myogenic transcription both by di-methylation of H3K9 on the myogenin promoter and methylation of MyoD protein (Ling *et al*., 2012, Figure 3.3.1.), the role of Sharp-1 in MyoD methylation was analysed by western blot.

Cells were transfected with MyoD and Sharp-1 and left untreated or treated with BIX-01294. Lysates were analysed for expression of endogenous G9a, MyoD and Myc-Sharp-1. Cell lysates were then immunoprecipitated with MyoD and analysed by western blot to determine MyoD methylation and its association with G9a in the presence of Sharp-1. G9a association with MyoD was increased and correspondingly MyoD methylation. Sharp-1-mediated increased in methylation of MyoD could be blocked by BIX-01294 (Figure 3.13.3.). Thus, Sharp-1 promotes G9a association with MyoD as well as MyoD methylation which can be reversed by BIX-01294.

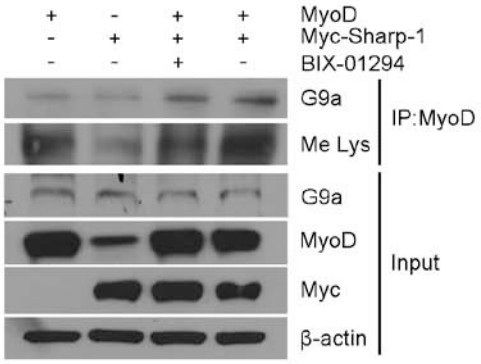

Figure 3.13.3 BIX-01294 blocks Sharp-1-mediated methylation of MyoD.

C2C12 cells were transfected with MyoD and/or Myc-Sharp-1. Transfected cells were treated with DMSO (control) or 2.5 μM BIX-01294. Cell lysates were analysed by western blot with anti-G9a, anti-MyoD or anti-c-Myc antibody. β-actin was used as a internal control. The MyoD immunoprecipitates were analysed by western blot with methyl lysine (Me Lys) and G9a antibodies.

3.13.4. Inhibition of G9a activity rescues Sharp-1-mediated block of differentiation

To determine the role of G9a in Sharp-1-dependent block of myogenesis, C2C12 cells overexpressing pBabe and pBabe-Sharp-1 were treated with DMSO (control) or with 2.5 μM of BIX-01294 and cultured for differentiation assays. Cells were fixed and immunostained with MHC primary antibody, followed by Texas-red secondary antibody. Sharp-1 expressing cells showed lesser number of MHC-positive myotubes formed as compared to control cells and BIX-01294-treated Sharp-1 expressing cells (Figure 3.13.4.A). Therefore, G9a methyltransferase activity is involved in Sharp-1-mediated inhibition of myotube formation and thus, the differentiation defect can be rescued with BIX-01294. Calculation of the myogenic index revealed a significant recovery of MHC-positive myotubes in BIX-01294 treated cells overexpressing Sharp-1 (Figure 3.13.4.B). Cell lysates were collected and analysed by western blot for myogenic differentiation markers, myogenin and troponin T. β-actin was used as an internal control. The results showed that inhibition of G9a activity restored expression of myogenic markers myogenin and troponin T (Figure 3.13.4.C).

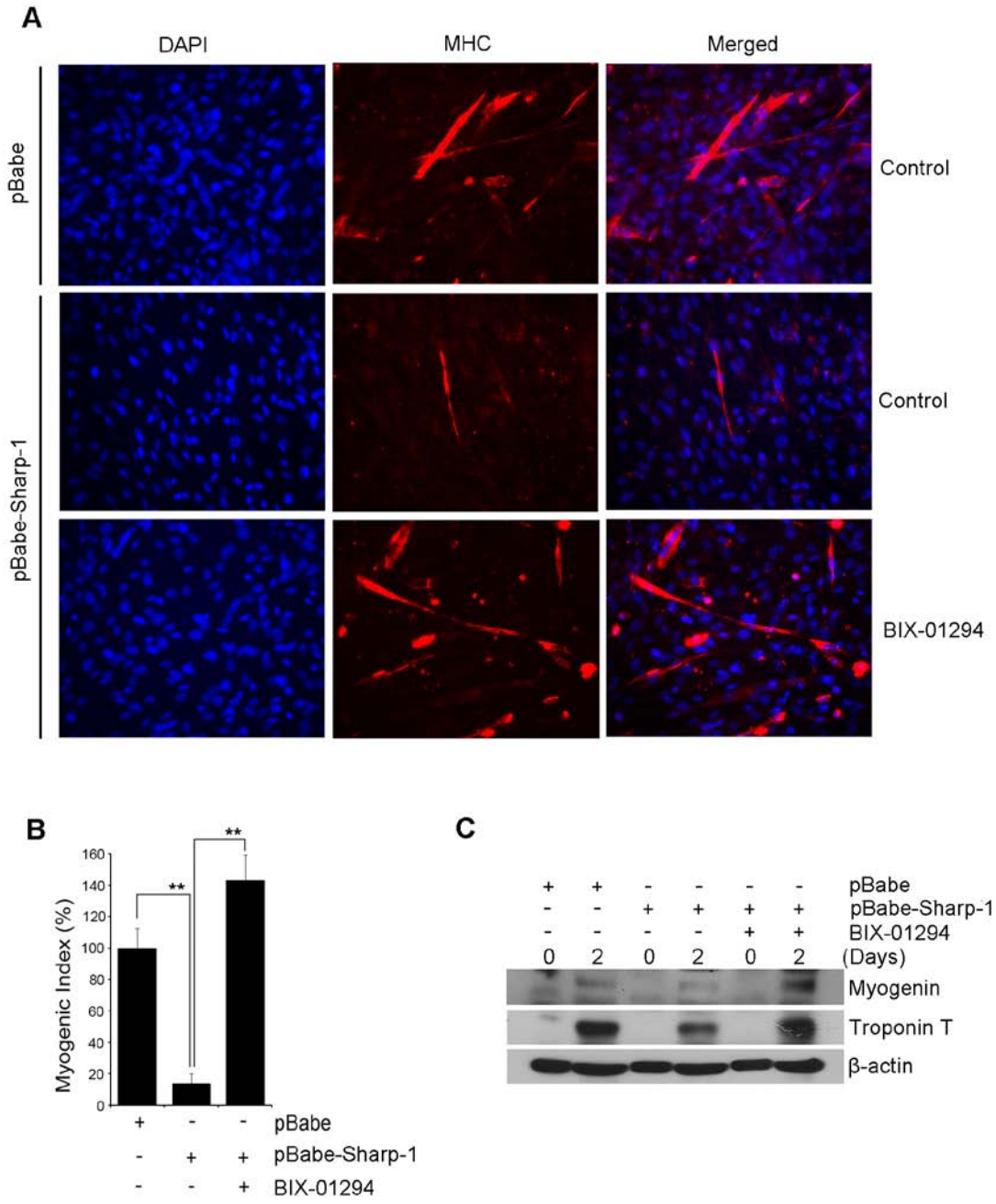

Figure 3.13.4 BIX-01294 blocks Sharp-1-mediated inhibition of differentiation and expression of muscle-specific genes.

pBabe- (vector control) or pBabe-Sharp-1-expressing cells were treated with DMSO (control) or 2.5 μM BIX-01294 and induced to differentiate for two days. Myotube formation was assessed by immunofluorescence staining with anti-myosin heavy chain antibody (MHC, red). Nuclei were stained with DAPI (blue). The images were

merged and captured using fluorescence microscope with 20x magnification (A). The extent of cell differentiation was determined by myogenic index. The mean value with standard deviation (error bar) obtained was expressed as percentage of the control (pBabe). P-values were calculated using Student's t-test and indicated as ** ($p < 0.01$, highly significant) (B). Day 0 and 2 cells lysates were analysed by western blot for myogenic marker myogenin and troponin T. β-actin was loaded as an internal control (C). These experiments were conducted at least two times with similar results.

3.14. siRNA knockdown of G9a rescues Sharp-1-mediated inhibition of myogenesis

3.14.1. siRNA-mediated knockdown of G9a rescues Sharp-1-induced inhibition of differentiation

A higher concentration of BIX-01294 has been reported to inhibit other methyltransferase such as GLP (Kubicek *et al*, 2007, Link *et al*., 2009). To ascertain if the differentiation defects induced by Sharp-1 was mediated through G9a, differentiation assays were performed with siRNA knockdown of G9a (siG9a). Sharp-1-overexpressing myoblast cells were transfected with siRNA or siG9a. Cells were cultured in GM and then DM to induce cellular differentiation for two days. Cell lysates were collected and subjected to western blot analysis for G9a expression. Western blot analysis showed that both G9a expression levels were clearly reduced with G9a knockdown (Figure 3.14.1.A). Upper band and lower band correspond to the long isoform and short isoform of G9a, respectively. Both control and siG9a cells were differentiated, and analysed for MHC-positive myotubes by immunofluorescence with anti-MHC antibody. G9a knockdown resulted an increased in the number of MHC-positive myotubes in Sharp-1-expressing cells (Figure 3.14.1.B). Thus, Sharp-1-mediated repression of myogenesis can be overcome by reducing G9a expression

during myoblast differentiation. This data further confirms that Sharp-1-represses myogenic differentiation through association with G9a. Myogenic index was determined to quantify the extent of recovery. A significant 7-fold increase in MHC-positive myotubes was apparent after reducing G9a expression (Figure 3.14.1.C).

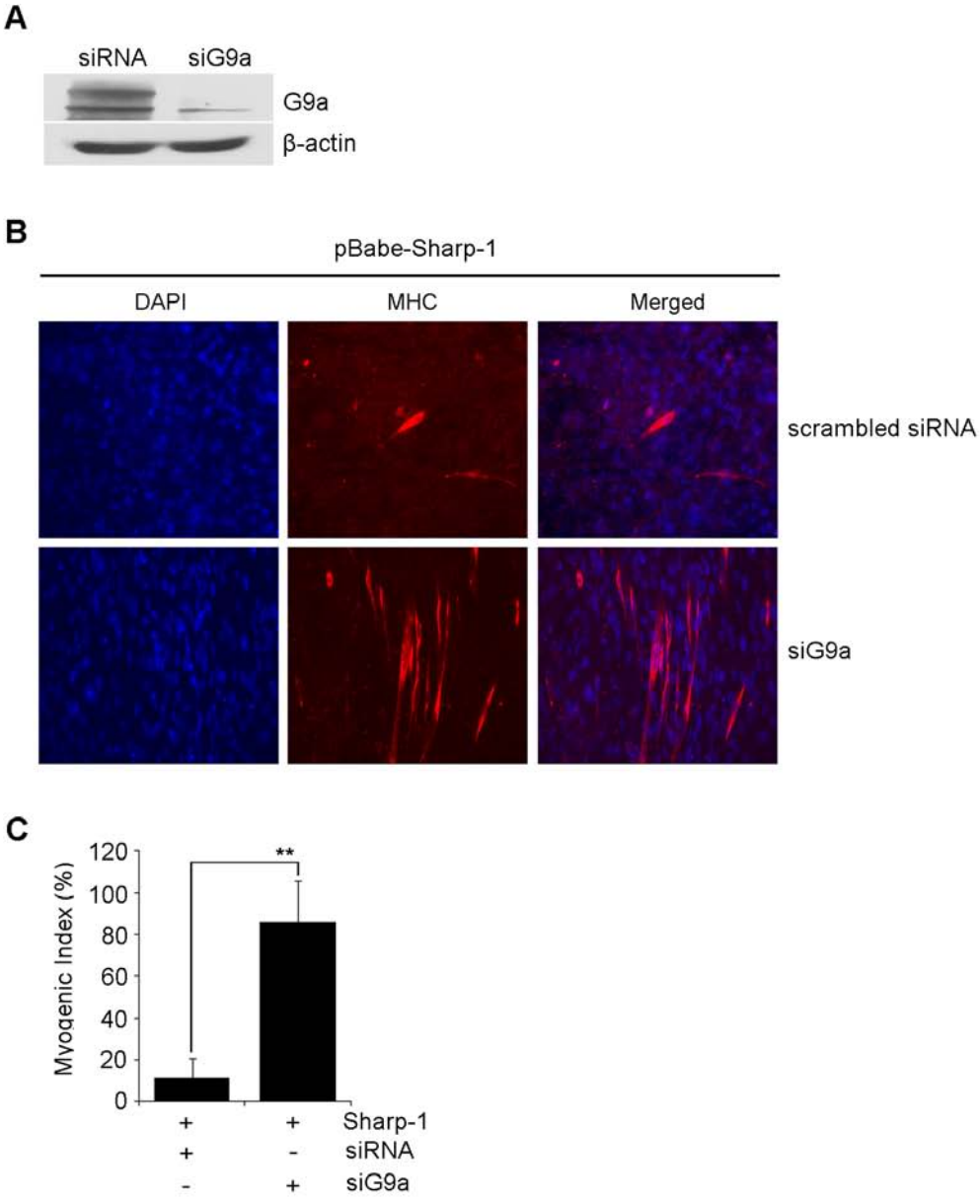

Figure 3.14.1 siRNA-mediated knockdown of G9a rescues Sharp-1-induced inhibition of differentiation.

pBabe-Sharp-1-expressing C2C12 cells were transfected with scrambled siRNA (control) or siRNA targeting G9a (siG9a). Cell lysates were analysed by western blot for G9a protein expression (A). Cells were induced to differentiate for two days and examined for myogeic differentiation. Cells were immunostained with anti-MHC antibody (red) and nuclei were stained with DAPI (blue). The images were merged and captured under a fluorescence microscope with 20x magnification (B). Myogenic index of pBabe-Sharp1 with scrambled siRNA or siG9a were determined as ratio of MHC-positive nuclei over total nuclei (at least 600 nuclei scored in each field). Values were shown as means with standard deviation (error bar). P-values were calculated using Student's t-test and indicated as ** ($p < 0.01$, very significant) (C). The assay was repeated twice with similar results observed.

3.14.2. Inhibition of G9a expression rescues Sharp-1-induced inhibition of MyoD target genes

The reduction of G9a expression in Sharp-1 expressing cells restored differentiation, the expression of myogenic markers was examined. Lysates from siRNA and siG9a cells were subjected to western blot analysis. In Sharp-1 expressing cells with G9a knockdown, the expression of both myogenic differentiation markers, myogenin and troponin T was increased (Figure 3.14.2). Together, data suggested that Sharp-1 inhibition of myogenesis is mediated through G9a.

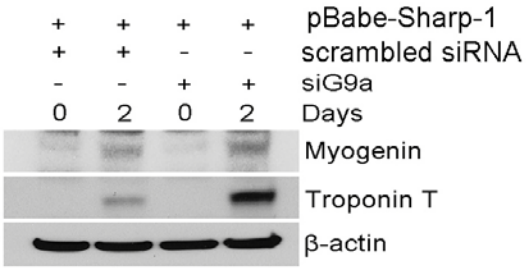

Figure 3.14.2 Inhibition of G9a expression rescues Sharp-1-induced inhibition of MyoD target genes.

pBabe-Sharp-1-expressing C2C12 cells were transfected with scrambled siRNA (control) or siRNA targeting G9a (siG9a). Cell lysates were analysed by western blot for myogenic differentiation marker myogenin and troponin T. β-actin was used as an internal control.

3.15. Re-expression of MyoD rescues Sharp-1-mediated inhibition of myogenesis

3.15.1. MyoD(K104R) exhibits increased activity in rescuing Sharp-1-mediated inhibition of differentiation

To examine whether MyoD(K104R) is indeed refractory to inhibition, C2C12 cells expressing Sharp-1 were transfected with pCS2 control vector, MyoD or MyoD(K104R). Cells were cultured in GM, followed by DM to induce differentiation for three days. The expression of MyoD and MyoD(K104R) in pBabe-Sharp-1-expressing cells were determined by western blot (Figure 3.15.1.A). The cells were differentiated and stained with MHC antibody. Consistent with the previous data, MyoD(K104R) was more effective than MyoD in rescuing myogenic differentiation in Sharp-1-overexpressing cells (Figure 3.15.1.B). Thus, re-expressing MyoD rescues myotube formation in Sharp-1-expressing cells and the effect is enhanced with expression of MyoD(K104R) which is not methylated and repressed by Sharp-1-

mediated recruitment of G9a. Myogenic index in Sharp-1 expressing cells rescued by MyoD and MyoD(K104R) was assessed. Rescue of MHC-positive myotubes in Sharp-1 expressing cells was significantly better with MyoD(K104R) compared with MyoD (Figure 3.15.1.C).

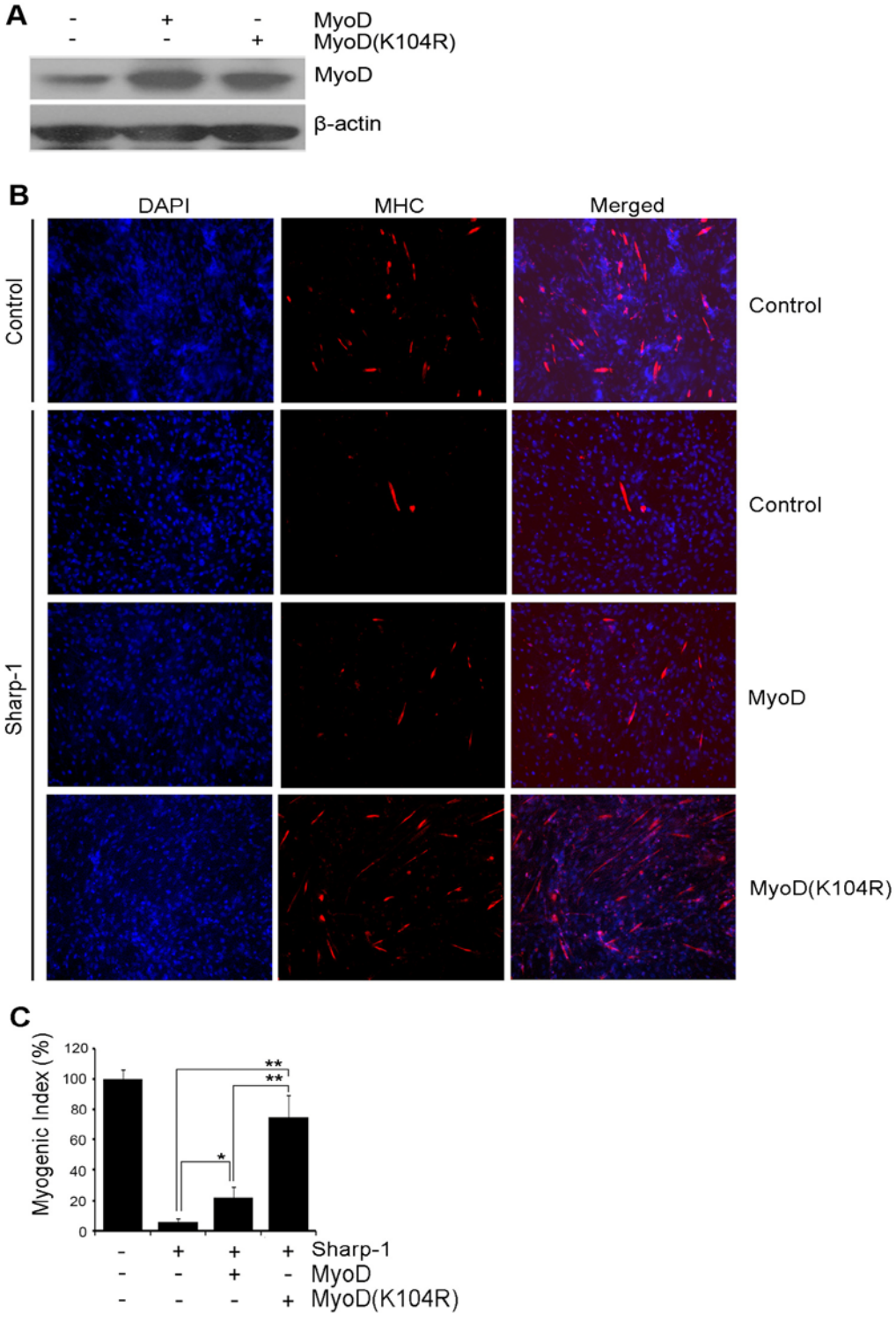

Figure 3.15.1 MyoD(K104R) is more effective than MyoD at restoration of the myotube formation in Sharp-1 overexpressing cells.

Sharp-1-expressing cells were transfected with pCS2 (control), MyoD and MyoD(K104R) and induced to differentiate for three days. Cell lysates were analysed by western blot for the expression MyoD, MyoD(K104R) mutant. β-actin was used as a loading control (A). Myogenic differentiation was assessed by immunofluorescence staining with anti-MHC antibody (red). Nuclei were counterstained with DAPI (blue). Images were microscope with a 10x magnification (B). The extent of the myotube formation was evaluated with myogenic index. The mean value obtained was presented as a percentage of control (C2C12 cells transfected with pCS2 only) with standard deviation (error bar). P-values were calculated using Student's t-test (* $p<0.05$, significant, ** $p<0.01$, highly significant) (C). Similar results were observed from at least two independent experiments.

3.15.2. Re-expression of MyoD(K104R) results in higher troponin T expression in Sharp-1-overexpressing cells

To examine the muscle protein expression, cell lysates were collected and analysed for the expression of muscle differentiation genes. Consistent with a block in differentiation, Sharp-1 expressing cells showed less troponin T expression than control cells. Consistent with the MHC-positive cells and myogenic index, earlier and increased troponin T expression was seen in cells transfected with MyoD(K104R), compared to MyoD (Figure 3.15.2). Therefore, increasing the expression of MyoD and MyoD(K104R) restore expression of muscle differentiation genes and overcome the differentiation defects mediated by Sharp-1. Since MyoD(K104R) is not methylated and its activity not inhibited by Sharp-1 associated with G9a, the rescue of differentiation is better with this mutant.

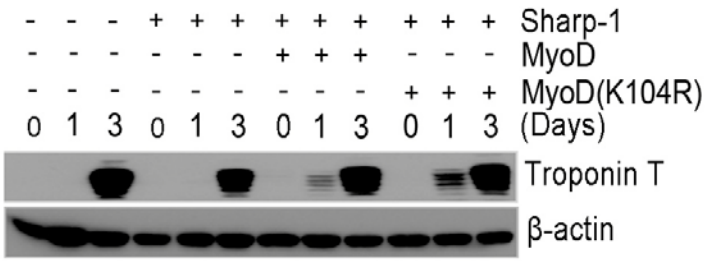

Figure 3.15.2 Re-expression of MyoD(K104R) results in higher troponin T expression in Sharp-1-overexpressing cells.

Sharp-1-expressing cells were transfected with pCS2 (control), MyoD and MyoD(K104R) and induced to differentiate for three days. Cell lysates were collected from cells at day 0, 1, 3 and analysed for terminal differentiation marker troponin T. β-actin was used as a loading control.

3.15.3. MyoD or MyoD(K104R) do not alter H3K9me2 on myogenin promoter during differentiation

During the inhibition of myogenic differentiation, besides methylation of MyoD activity, H3K9me2 on myogenin promoter can also repress transcription of muscle genes. H3K9me2 on myogenin promoter was assessed by ChIP assay in Sharp-1expressing cells that were transfected with MyoD and MyoD(K104R). Cell lysates were immunoprecipitated with H3K9me2 antibody and amplified with primers specific to myogenin promoter. In Sharp-1 overexpressing cells, re-expression of MyoD and MyoD(K104R) resulted in similar levels of H3K9me2 levels on myogenin promoter (Figure 3.15.3). This indicates the methylation of MyoD plays a greater role in Sharp-1-mediated repression of differentiation.

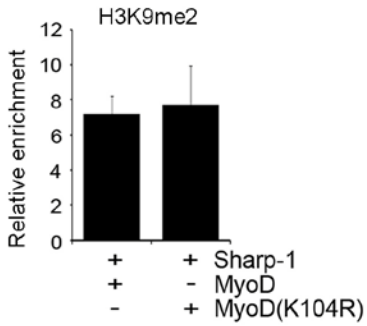

Figure 3.15.3 Re-expression of MyoD or MyoD(K104R) does not alter H3K9me2 on myogenin promoter in Sharp-1-expressing cells.

Sharp-1-expressing C2C12 cells were transfected with MyoD and MyoD(K104R) mutant and induced to differentiate for two days. Cell lysates were used for ChIP analysis to determine the H3K9me2 on the myogenin promoter. Chromatin was analysed by QPCR using primers for the myogenin promoter and β-actin gene (an internal control). Values obtained were plotted as means with standard deviation (error bar). The ChIP experiment was conducted twice.

Chapter 4

Discussion

4. Discussion

Skeletal muscle cell differentiation in embryonic development and post-natal muscle regeneration is positively regulated by MRFs as well as negatively controlled by repressors such as Sharp-1. The inhibitory activity of Sharp-1 occurs through several mechanisms. One way is through the interaction with MyoD and E-protein, thus preventing formation of MyoD-E protein complexes from activating muscle genes. However, the mechanisms of Sharp-1 in myogenesis are not well defined. In this study, I have identified lysine methyltransferase G9a as a novel transcriptional co-repressor that directly associates with Sharp-1, and is critical for Sharp-1-mediated repression of MyoD activity and muscle differentiation. Similar to Sharp-1, G9a itself has an inhibitory role in skeletal myogenesis. I demonstrated that both Sharp-1 and G9a expression decline as cells undergo terminal differentiation. In addition, overexpression of Sharp-1 or G9a alone blocks muscle differentiation. These results are consistent with a previous study which demonstrated that Sharp-1 expression downregulated during differentiation and the expression of muscle genes required for myoblast differentiation are transcriptionally repressed in Sharp-1-expressing cells (Azmi *et al*., 2004).

Studies thus far have not shown any role of G9a in muscle cells. Hence, for the first time, I found that G9a inhibit muscle differentiation and its repression is dependent upon its lysine methyltransferase activity, conferred by the SET domain. Deletion of the catalytic domain in G9a abrogates inhibition of myogenic differentiation. A methyltransferase inhibitor BIX-01294 was shown to selectively target G9a reduce H3K9me2 levels in mammalian cells at low micromolar concentration (Kubicek *et al*., 2007, Chang *et al*., 2009). Notably, inhibition of G9a activity with BIX-01294 reduces G9a-mediated transcriptional repression of muscle differentiation genes,

confirming that the methyltransferase activity of G9a is required for inhibition of muscle differentiation program. Although myogenin expression was induced after long exposure with BIX-01294 in myoblasts, no myotubes developed which implies the presence of other inhibitors that co-function with G9a to prevent myoblast differentiation. Although a low concentration of BIX-0129 was used, the specificity may still be an issue since it can also inhibit GLP (Chang *et al*., 2009, Shinkai *et al*., 2011). Hence, countering this specificity problem, a more specific drug UNC0638 or siRNA-mediated knockdown of G9a are alternatively used in the assay with the same results obtained (data not shown here but can be found in Ling *et al*., 2012).

Apart from epigenetic modulation of histones, G9a is also capable of binding to and methylating MyoD at K104 which attenuates its transcriptional activity (data not shown here but can be found in Ling *et al*., 2012). Consistent with this finding, results showed that re-expression of MyoD(K104R) which is incapable of being methylated by G9a, rescues the differentiation block imposed by G9a better than wild type MyoD.

Besides repression of muscle-specific gene expression, I present evidence that G9a also inhibits p21 expression and facilitates cell cycle progression both in proliferating and differentiating myoblasts. Many reports have previously demonstrated that G9a downregulates p21 gene in non-muscle cells, resulting in increased expression of S-phase genes and promoting high mitotic index (Gyory *et al*., 2004, Nishio and Walsh, 2004, Kim *et al*., 2008). In myogenesis, however, the upregulation of p21 by MyoD is necessary to promote permanent cell cycle exit prior to myoblast differentiation (Guo *et al*., 1995, Hawke *et al*., 2003). Hence, I hypothesize that G9a mediates H3K9me2 on p21 promoter restricting its gene expression and causing a delay in cell cycle exit. Thus, myoblasts that fail to exit the cell cycle will resist terminal differentiation. This

is another possible mechanism by which G9a may control cell-cycle regulators and lead to an impairment of muscle cell differentiation.

I also showed that overexpression of Sharp-1 in myoblasts results in differentiation defects similar to those observed with overexpression of G9a. Sharp-1 increases H3K9me2 and decreases H3K9K14ac levels on myogenin promoter, as well as increases methylation of MyoD. These findings suggest that H3K9me2 plays a role in Sharp-1-mediated silencing of gene expression. G9a is a transcriptional co-repressor that is responsible for H3K9me2 but it does not contain a DNA-binding sequence and needs to be recruited by a transcription factor. Following, it was discovered that the physical interaction between Sharp-1 and G9a is required in Sharp-1-mediated repression. The protein interaction studies establish that G9a through its ankyrin domain interacts directly with Sharp-1, via its C-terminus spanning amino acids 173 to 265. The G9a interaction with Sharp-1 is important as the repressive effect of Sharp-1 on MyoD transcriptional activity was enhanced in the presence of G9a but not G9aΔANK mutant which cannot associate with Sharp-1. In addition to their direct association, Sharp-1-inhibitory mechanisms also require G9a methyltransferase activity. This observation is clearly supported by experiments showing that the methyltransferase inhibitor BIX-01294 is able to overcome Sharp-1-mediated repression of MyoD transcriptional activity and muscle gene expression. Moreoever, siRNA-induced knockdown of G9a expression or inhibition of G9a activity by BIX-01294 partially reverses Sharp-1-mediated inhibitory effect on muscle differentiation. Furthermore, exogenous expression of MyoD(K104R) rescues Sharp-1-mediated differentiation defects better than wild type MyoD. Collectively, my data demonstrates that Sharp-1-mediated inhibition of myogenesis is at least partially dependent on recruitment of G9a at the muscle promoters and MyoD. The mechanism

involves both methylation of MyoD and H3K9me at MyoD-target promoter gene by G9a.

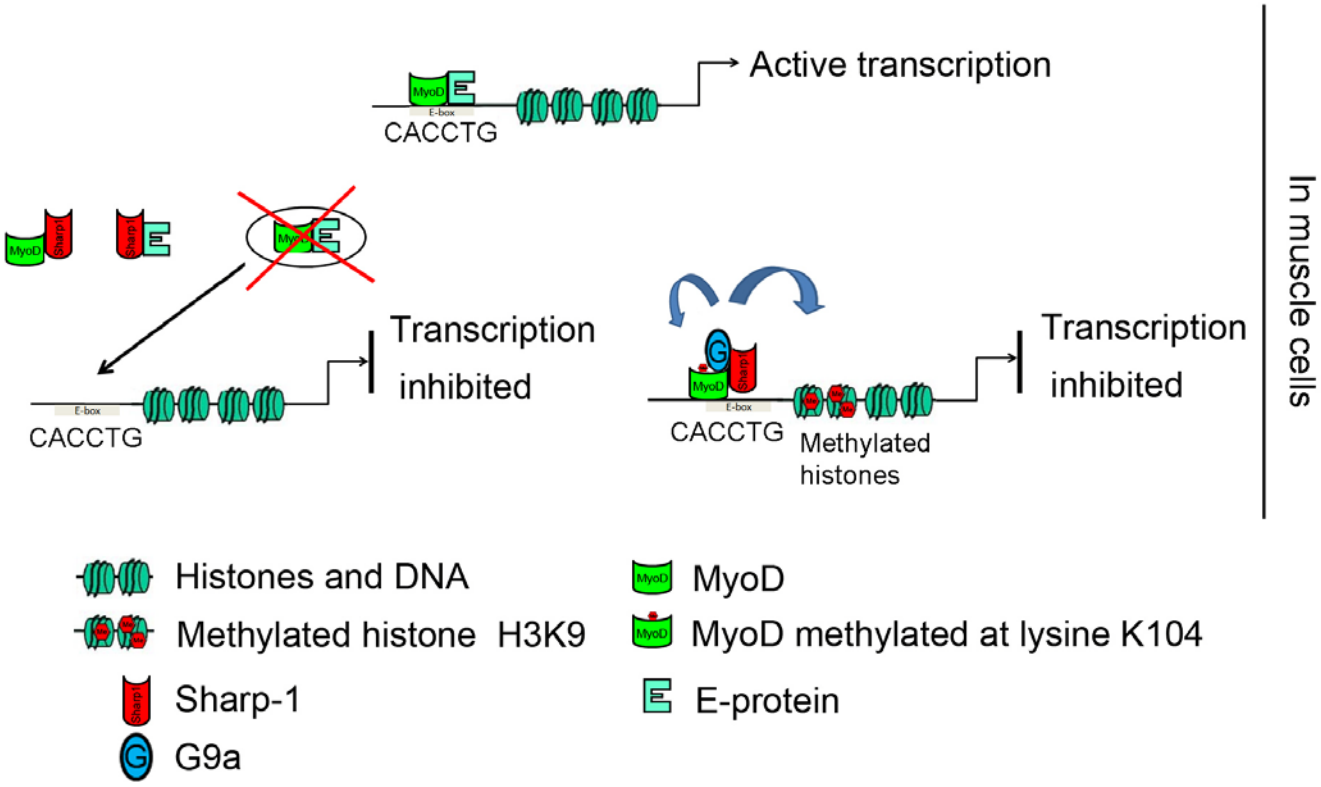

Figure 4.1 A model for Sharp-1-dependent inhibition of transcription.

Apart from my research study in skeletal muscle, many previous works on non-muscle cell type have established G9a as an important epigenetic regulator where it limits the access of regulatory proteins to DNA, thus posing serious restraints to biological processes such as replication and transcription. A disruption of the balance in the biological processes can cause several major pathologies including syndromes involving chromosomal instabilities, and mental retardation. For example, a report demonstrated that the maintenance of CpG methylation in Prader Willi Syndrome

requires G9a-mediated H3K9 methylation (Xin *et al*., 2003). Another report revealed that G9a has a crucial role in drug addiction, where they showed that repeated cocaine administration reduced global levels of H3K9 dimethylation and promoted cocaine preference in mice (Maze *et al*., 2010). Lee and his group found that G9a functions both as a co-repressor for specific transcription factors and co-activators for nuclear receptors, co-operating synergistically with gene expression punctually (Lee *et al*., 2006). Work from Shinkai group showed that G9a-deficient embryos die during early development with drastic loss of H3K9me levels in the euchromatic regions and increased cell death. Their findings suggest that G9a is required for the efficient repression of developmentally regulated genes during embryogenesis (Tachibana *et al*., 2008).

For the past years, researchers also study the mechanism of G9a in the carcinogenesis and development of human malignant tumor. For example, recent studies provided insight into how RelB recruits G9a to initiate gene silencing, resulting in phenotype associated with severe systemic inflammation in human (Chen *et al*., 2009). A researcher showed that G9a also plays an important role in the hypoxia-induced H3K9me2, which would inhibit the expression of several genes that would likely lead to solid tumor progression (Chen *et al*., 2006). In human breast cancer, G9a interacts with Snail and is critical for Snail-mediated E-cadherin repression (Dong *et al*., 2012). In addition, histone modifications by G9a are involved in regulating centrosome duplication presumably through chromatin structure rather than through affecting gene expression in cancer cells (Kondo *et al*., 2008). Another study suggests that G9a is a potential inhibitory target for cancer treatment where their data revealed a new methylation site within p53 mediated by the methylases G9a (Huang *et al*., 2010).

Many data confirmed the association between the presence of G9a and cancer, however, there are no reports on G9a in myopathies. Although there have been no reports on G9a role in myogenesis and myopathy, other methyltransferase such as Suv39h1/KMT1A has been shown to impair MyoD function and arrest myogenic differentiation in alveolar rabdomyosarcoma (Lee *et al*., 2011). Another report revealed that SUV39H1–mediated H3K9 tri-methylation at tandem repeat sequence D4Z4 seen in normal cells is lost in Facioscapulohumeral dystrophy, an autosomal dominant muscular dystrophy (Zeng *et al*., 2009). However, it is still unclear how transcriptional repressor Suv39H1 is associated with MyoD and the molecular mechanism of the methyltransferase that caused the blockade of differentiation remains to be determined. This research work therefore highlights the possibility of another methyltransferase and a mechanism contributing to the failure of myogenic differentiation, paving the way to new therapeutic options.

As for Sharp-1, it has been documented to be involved in the regulation of differentiation and growth of several cell types beside skeletal muscle. One study found that Sharp-1 plays a role in terminal neuronal differentiation (Rossner *et al*., 1997) while another study demonstrated that Dec2/Sharp-1 regulates TH2 lineage commitment and cell differentiation (Yang *et al*., 2009a). Sharp-1 blocks adipocyte differentiation by interacting with and inhibiting transcriptional activity of both CEBP/α and CEBP/β (Gulbagci *et al*., 2009). A genetic study found that a mutation in Sharp-1 gene may affect sleep time in mice (He *et al*., 2009). Researchers have also explored the role of Sharp-1 in tumorigenesis and cancer progression. It has been shown that Sharp-1 suppresses p53-mediated apoptotic activities and mediates cellular response to DNA damage (Liu *et al*., 2010). Clinical data revealed that Sharp-

1 suppresses breast cancer metastasis through degradation of hypoxia-inducible factors (Montagner *et al*., 2012).

Concerning muscle development, it has been documented that Sharp-1 interacts with and inhibits MyoD transcriptional activity, resulting in myogenic differentiation defects (Azmi *et al*., 2004). Similar to these findings, it was found that Sharp-1 is highly expressed in inclusion body myositis, an inflammatory muscle disease (Morosetti *et al*., 2006). While many studies provide strong evidence that Sharp-1 expression correlated with pathologies, however its repressive mechanisms in skeletal myogenesis are not well defined. Therefore, the study here has provided a platform for understanding the molecular mechanisms of Sharp-1 in inhibiting myogenesis and its possible link with myopathies. Observations from several pathological studies, together with findings reported here raise the possibility of a therapeutic potential of targeting G9a activity in myopathies with elevated Sharp-1 levels.

Conclusion and future studies

Conclusion and future studies

This research study elucidates the mechanism by which Sharp-1 mediates transcriptional silencing of muscle genes in myoblasts via its association with the co-factor G9a. This information is useful towards understanding the development and regeneration process in skeletal muscle. However, there are some unknowns in the present study and require further investigation.

Besides repressing myogenic differentiation genes, G9a participates in the downregulation of p21 expression which contributes to maintenance of myoblast proliferation and probably results in delaying cell differentiation. At this stage, it is not known if G9a is involved in regulating other cell cycle genes and raises the question of whether G9a has any important role in controlling the cell cycle exit and consequently differentiation as well as possibly regulating cellular quiescence. As such, the function of G9a in cell cycle progression remains to be determined. In addition, G9a and GLP protein complexes mediate H3K9 methylation and DNA methylation to silence gene transcription (Tachibana *et al*., 2008, Collins *et al*., 2010) which raises the possibility of the role of GLP and its co-operation with G9a in the regulation of myogenesis. Furthermore, it has been shown that G9a indirectly methylates DNA through recruitment of Dnmtase (Litman *et al*., 2008) and G9a knockout cells demonstrates a significant reduction of DNA methylation (Litman *et al*., 2008, Xin *et al*., 2003, Esteve *et al*., 2006, Dong *et al*., 2008). Thus, it will be interesting to find out if Dnmtase are also involved in Sharp-1-mediated block of muscle cell differentiation and development. Hence, further investigations on the mechanism of Sharp-1 and G9a in regulating myogenesis are necessary as many G9a-related questions remain.

More in-depth work will involve analysing the role of G9a in muscle development and regeneration *in vivo*, as well as identification of target genes and determining if its expression or activity is perturbed in muscle disorders such as muscular dystrophies. Muscle regeneration depends on the activation and proliferation of satellite cells. It is not known if G9a is also involved in the activation of satellite cells and muscle regeneration since it is likely to be involved in the proliferation of muscle cells. Hence, further in-depth studies are necessary to determine if G9a is involved in skeletal muscle regeneration.

Together, this research work and future work will provide new insights into Sharp-1-dependent regulation of myogenesis and identify epigenetic mechanisms which could be targeted in myopathies characterized by elevated Sharp-1 levels. These will be useful towards the development of new therapeutic strategies in the treatment of the human muscle diseases.

Table I. Primers for RT-PCR.

Gene	Forward primer	Reverse primer	TM (°C)	No. of cycles
mG9a (mouse G9a)	GTCGAAGCTCTAGCTG AACAG	CCTGAGGAACCCACAC CATT	55	28
mhG9a (mouse, human G9a)	GAGGAGTGGGAGACGG TGGTG	GCCATTTCTTCTTGGCC TTG CGCCGG	65	28
MyoD	GCCCGCGCTCCAACTGC TCTGAT	CCTACGGTGGTGCGCC CTCTGC	58	25
Myogenin	GGGCCCCTGGAAGAAA AG	AGGAGGCGCTGTGGGA GTT	58	25
36B4	CAGCTCTGGAGAAACT GCTG	GTGTACTCAGTCTCCAC AGA	55	19

TABLE II. Primers for Q-PCR.

Gene	Forward primer	Reverse primer	TM (°C)
mG9a (mouse G9a)	TCGGGCAATCAGTCAGACA G	TGAGGAACCCACACCATT CAC	60
mSharp-1 (mouse Sharp-1)	AACACTGGGGCATTTGGAG A	TGGACCGGCGATTTCAGA G	60
GAPDH	AGGAGCGAGACCCCACTA ACAT	GTGAAGACACCAGTAGAC TCCACG	60
Myogenin promoter	TGGCTATATTTATCTCTGG GTTCATG	GCTCCCGCAGCCCCT	60
β-actin	GCTTCTTTGCAGCTCCTTCG TTG	TTTGCACATGCCGGAGCC GTTGT	60

TABLE III. Primers for siRNA.

Smart pool siRNA sequences	sequence	matching sequence (in human)
Non-targeting siRNA -1	UGGUUUACAUGUCG ACUAA	
Non-targeting siRNA -2	UGGUUUACAUGUUG UGUGA	
Non-targeting siRNA -3	UGGUUUACAUGUUU UCUGA	
Non-targeting siRNA -4	UGGUUUACAUGUUU UCCUA	
siG9a-1	UAACAAGGAUGGCG AGGUU	CCAAGAAGAAATG GCGAA
siG9a-2	CCAUGAACAUCGAC CGCAA	CAGGACAGGTGGA CGTCAA
siG9a-3	CAGGACAGGUGGAC GUCAA	CCATGAACATCGAT CGCAA
siG9a-4	CCAAGAAGAAAUGG CGGAA	CAACAAGGATGGA GAGGTG

TABLE IV. Primers for cloning Sharp-1 mutants.

Gene	Forward primer	Reverse primer	TM (°C)
Myc-Sharp-1 ΔO (1-128)a a	CGAGGGATCCATGG AGCAGAAACTCATC TCTGAAGAGGATCT GGACGAAGGAATC CCTC	ATCCAAGTCGGCCTGG ACCGG	55
Myc-Sharp-1 ΔO (174-280)a a	CTGACGCCACAGGT GCCCTCC	GCGAAGGGCGCGCCCC CGCCA	61
Myc-Sharp-1 ΔC (1-276)a a	CGAGGGATCCATGG AGCAGAAACTCATC TCTGAAGAGGATCT GGACGAAGGAATC CCTC	GCGCGAATTCGGG CCCAGGAGC GCGC	60

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